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(54) Title: ANTISENSE INHIBITION OF ras GENE WITH CHIMERIC AND ALTERNATING OLIGONUCLEOTIDES

(57) Abstract

Compositions and methods are provided for the modulation of expression of the human ras gene in both the normal and activated forms. Oligonucleotides are provided that have methylene(methylimino) linkages alternating with phosphorothioate or phosphodiester linkages. Further oligonucleotides are provided that have a first region having a methylene(methylimino) linkage alternating with a phosphorothioate or phosphodiester linkage and a second region having phosphorothioate linkages. Such oligonucleotides can be used for diagnostics as well as for research purposes including methods for diagnosis, detection and treatment of conditions arising from the activation of the H-ras gene.

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ANTISENSE INHIBITION OF *ras* GENE WITH CHIMERIC AND ALTERNATING OLIGONUCLEOTIDES

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application No: 411,734, filed April 13, 1995; PCT Application PCT/US93/09346, filed October 1, 1993; U.S. Patent Application No: 715,196, filed June 14, 1991, now abandon; U.S. Patent Application No: 958,134, filed October 5, 1992, now abandon; U.S. Patent Application No. 292,086, filed July 19, 1994, that issued as U.S. Patent 5,582,986; U.S. Patent Application No: 08/007,996, filed January 21, 1993, now abandon; U.S. Patent Application 297,248, filed July 26, 1994, that issued as U.S. Patent 5,576,208; U.S. Patent Application 703,619, filed May 21, 1991, that issued as United States patents 5,378,825; U.S. Patent Application 040,903, filed March 31, 1993, that issued as U.S. Patent 5,386,023; U.S. Patent Application 040,526, filed on March 31, 1993, that issued as U.S. Patent 5,489,677; U.S. Patent Application 174,379, filed December 28, 1993, that issued as U.S. Patent 5,541,307; U.S. Patent Application 040,933, filed March 31, 1993, now abandon; U.S. Patent Application 300,072, filed September 2, 1994, that issued U.S. Patent 5,618,704; U.S. Patent Application 039,979, filed March 30, 1993, now abandon; U.S. Patent Application 395,168, filed February 27, 1995, that issued as U.S. Patent 5,623,070; U.S. Patent Application 814,961, filed December 24, 1991, now abandon; PCT Patent Application PCT/US92/11339, filed December 23, 1992; and U.S. Patent Application 244,993, filed

June 21, 1994, that issued as U.S. Patent 5,623,065; and U.S. Patent Applicatin 468,037, filed June 6, 1995; each of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

This invention relates to compositions and methods for the inhibition of expression of the ras gene, a naturally occurring gene which occasionally converts to an activated form that has been implicated in tumor formation. This invention is also directed to the specific inhibition of expression of the activated form of the ras gene. This invention is further directed to the detection of both normal and activated forms of the ras gene in cells and tissues, and can form the basis for research reagents and kits both for research and diagnosis. Furthermore, this invention is directed to treatment of such conditions as arise from activation of the ras gene. This invention also relates to stabilized oligonucleotides for inhibition of expression of the ras gene, to such oligonucleotides which have been further modified to enhance their affinity for the ras RNA target, and to such oligonucleotides which have been still further modified to yield sequence-specific elimination of the ras RNA target.

BACKGROUND OF THE INVENTION

Alterations in the cellular genes which directly or indirectly control cell growth and differentiation are considered to be the main cause of cancer. There are some thirty families of genes, called oncogenes, which are implicated in human tumor formation. Members of one such family, the ras gene family, are frequently found to be mutated in human tumors. In their normal state, proteins produced by the ras genes are thought to be involved in normal cell growth and maturation. Mutation of the ras gene, causing an amino acid alteration at one of three critical positions in the protein product, results in conversion to a form which is implicated in tumor formation. A gene having such a mutation is said to be "activated." It is thought that such a point

mutation leading to ras activation can be induced by carcinogens or other environmental factors. Over 90% of pancreatic adenocarcinomas, about 50% of adenomas and adenocarcinomas of the colon, about 50% of adenocarcinomas of the lung and carcinomas of the thyroid, and a large fraction of malignancies of the blood such as acute myeloid leukemia and myelodysplastic syndrome have been found to contain activated ras oncogenes. Overall, some 10 to 20% of human tumors have a mutation in one of the three ras genes (H-ras, K-ras, or N-ras).

It is presently believed that inhibiting expression of activated oncogenes in a particular tumor cell might force the cell back into a more normal growth habit. For example, Feramisco et al., *Nature* **1985**, 314, 639-642, demonstrated that if cells transformed to a malignant state with an activated ras gene are microinjected with antibody which binds to the protein product of the ras gene, the cells slow their rate of proliferation and adopt a more normal appearance. This has been interpreted as support for the involvement of the product of the activated ras gene in the uncontrolled growth typical of cancer cells.

The H-ras gene has recently been implicated in a serious cardiac arrhythmia called long Q-T syndrome, a hereditary condition which often causes sudden death if treatment is not given immediately. Frequently there are no symptoms prior to the onset of the erratic heartbeat. Whether the H-ras gene is precisely responsible for long Q-T syndrome is unclear. However, there is an extremely high correlation between inheritance of this syndrome and the presence of a particular variant of the chromosome 11 region surrounding the H-ras gene. Therefore, the H-ras gene is a useful indicator of increased risk of sudden cardiac death due to the long Q-T syndrome.

There is a great desire to provide compositions of matter which can modulate the expression of the ras gene, and particularly to provide compositions of matter which specifically modulate the expression of the activated form of

the ras gene. It is greatly desired to provide methods of diagnosis and detection of the ras gene in animals. It is also desired to provide methods of diagnosis and treatment of conditions arising from ras gene activation. In addition, improved research kits and reagents for detection and study of the ras gene are desired.

Inhibition of oncogene expression has been accomplished using retroviral vectors or plasmid vectors which express a 2-kilobase segment of the Ki-ras protooncogene RNA in antisense orientation. Mukhopadhyay, T. et al. (1991) *Cancer Research* 51, 1744-1748; PCT Patent Application PCT/US92/01852 (WO 92/15680); Georges, R.N. et al. (1993) *Cancer Research*, 53, 1743-1746.

Antisense oligonucleotide inhibition of oncogenes has proven to be a useful tool in understanding the roles of various oncogene families. Antisense oligonucleotides are small oligonucleotides which are complementary to the "sense" or coding strand of a given gene, and as a result are also complementary to, and thus able to stably and specifically hybridize with, the mRNA transcript of the gene. Holt et al., *Mol. Cell Biol.* **1988**, 8, 963-973, have shown that antisense oligonucleotides hybridizing specifically with mRNA transcripts of the oncogene c-myc, when added to cultured HL60 leukemic cells, inhibit proliferation and induce differentiation. Anfossi et al., *Proc. Natl. Acad. Sci.* **1989**, 86, 3379-3383, have shown that antisense oligonucleotides specifically hybridizing with mRNA transcripts of the c-myb oncogene inhibit proliferation of human myeloid leukemia cell lines. Wickstrom et al., *Proc. Nat. Acad. Sci.* **1988**, 85, 1028-1032, have shown that expression of the protein product of the c-myc oncogene as well as proliferation of HL60 cultured leukemic cells are inhibited by antisense oligonucleotides hybridizing specifically with c-myc mRNA. United States Patent No: 4,871,838 (Bos et al.) discloses oligonucleotides complementary to a mutation in codon 13 of N-ras to detect said mutation. United States Patent No: 4,871,838 (Bos et al.) discloses

molecules useful as probes for detecting a mutation in DNA which encodes a ras protein.

In all these cases, instability of unmodified oligonucleotides has been a major problem, as they are subject to degradation by cellular enzymes. PCT/US88/01024 (Zon et al.) discloses phosphorothioate oligonucleotides hybridizable to the translation initiation region of the amplified c-myc oncogene to inhibit HL-60 leukemia cell growth and DNA synthesis in these cells. Tidd et al., *Anti-Cancer Drug Design* **1988**, 3, 117-127, evaluated methylphosphonate antisense oligonucleotides hybridizing specifically to the activated N-ras oncogene and found that while they were resistant to biochemical degradation and were nontoxic in cultured human HT29 cells, they did not inhibit N-ras gene expression and had no effect on these cells. Chang et al. showed that both methylphosphonate and phosphorothioate oligonucleotides hybridizing specifically to mRNA transcripts of the mouse Balb-ras gene could inhibit translation of the protein product of this gene *in vitro*. Chang et al., *Anti-Cancer Drug Design* **1989**, 4, 221-232; Brown et al., *Oncogene Research* **1989**, 4, 243-252. It was noted that T_m was not well correlated with antisense activity of these oligonucleotides against *in vitro* translation of the ras p21 protein product. Because the antisense oligonucleotides used by Chang et al. hybridize specifically with the translation initiation region of the ras gene, they are not expected to show any selectivity for activated ras and the binding ability of these oligonucleotides to normal (wild-type) vs. mutated (activated) ras genes was not compared.

Helene and co-workers have demonstrated selective inhibition of activated (codon 12 G→T transition) H-ras mRNA expression using a 9-mer phosphodiester linked to an acridine intercalating agent and/or a hydrophobic tail. This compound displayed selective targeting of mutant ras message in both Rnase H and cell proliferation assays at low micromolar concentrations. Saison-Behmoaras, T. et al., *EMBO J.* **1991**, 10, 1111-1118. Chang and co-workers disclose selective targeting of mutant H-ras message; this time the target was H-ras codon

61 containing an A→T transversion and the oligonucleotide employed was either an 11-mer methylphosphonate or its psoralen derivative. These compounds, which required concentrations of 7.5-150 μM for activity, were shown by immunoprecipitation to selectively inhibit mutant H-ras p21 expression relative to normal p21. Chang et al., *Biochemistry* **1991**, 30, 8283-8286.

Modified nucleotides which increase $\Delta\Delta G^{\circ}_{37}$ for base mismatches can be used to increase selectivity. It has been found that $\Delta\Delta G^{\circ}_{37}$ ranges from 1-2 kcal/mol for the most stable mismatches to 5-6 kcal/mol for the least stable mismatches. When possible, therefore, to maximize selectivity for the mutant target, mutations that generate stable mismatches (e.g., G→A) are less preferred than mutations that generate unstable mismatches (e.g., C→G, U→G, A→C). An example of this can be found in the autosomal dominant mutations associated with familial Alzheimer's disease. Three different point mutations of the β-amyloid precursor gene have been shown to cosegregate with this disease. These mutations include G→A ($\Delta\Delta G^{\circ}_{37}$ = +1.2 kcal/mol), G→T ($\Delta\Delta G^{\circ}_{37}$ = +3.9 kcal/mol), and T→G ($\Delta\Delta G^{\circ}_{37}$ = +6.3 kcal/mol)[2]. Goate et al., *Nature* **1991**, 349, 704-706; Murrel et al., *Science* **1991**, 254, 97-99; Chartier-Harlin et al., *Nature* **1991**, 353, 844-846. In this case, targeting the T→G mutation is believed to yield the greatest selectivity for mutant β-amyloid by an antisense oligonucleotide.

DNA oligonucleotides having unmodified phosphodiester internucleoside linkages or modified phosphorothioate internucleoside linkages are substrates for cellular RNase H; i.e., they activate the cleavage of target RNA by the RNase H. (Dagle, J.M, Walder, J.A. and Weeks, D.L., *Nucleic Acids Research* **1990**, 18, 4751; Dagle, J.M., Weeks, D.L. and Walder, J.A., *Antisense Research And Development* **1991**, 1, 11; and Dagle, J.M., Andracki, M.E., DeVine, R.J. and Walder, J.A., *Nucleic Acids Research* **1991**, 19, 1805). RNase H is an endonuclease that cleaves the RNA strand of RNA:DNA duplexes; activation of this enzyme therefore results in cleavage of the RNA target, and thus can greatly enhance the ability of antisense oligonucleotides to inhibit target RNA expression.

Walder et al. note that in *Xenopus* embryos, both phosphodiester linkages and phosphorothioate linkages are also subject to exonuclease degradation. Such nuclease degradation is detrimental since it rapidly depletes the oligonucleotide available for RNase H activation. PCT Publication WO 89/05358, Walder et al., discloses DNA oligonucleotides modified at the 3' terminal internucleoside linkage to make them resistant to nucleases while remaining substrates for RNAse H.

Attempts to take advantage of the beneficial properties of oligonucleotide modifications while maintaining the substrate requirements for RNase H have led to the employment of chimeric oligonucleotides. Giles, R.V. et al., *Anti-Cancer Drug Design* **1992**, 7, 37; Hayase, Y. et al., *Biochemistry* **1990**, 29, 8793; Dagle, J.M. et al., *Nucleic Acids Research* **1990**, 18, 4751; Dagle, J.M. et al., *Nucleic Acids Research* **1991**, 19, 1805. Chimeric oligonucleotides contain two or more chemically distinct regions, each comprising at least one nucleotide. These oligonucleotides typically contain a region of modified nucleotides that confer one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the RNA target) and an unmodified region that retains the ability to direct RNase H cleavage. This approach has been employed for a variety of backbone modifications, most commonly methylphosphonates, which alone are not substrates for RNAse H. Methylphosphonate oligonucleotides containing RNase H-sensitive phosphodiester linkages were found to be able to direct target RNA cleavage by RNase H *in vitro*. Using *E. coli* RNase H, the minimum phosphodiester length required to direct efficient RNase H cleavage of target RNA strands has been reported to be either three or four linkages. Quartin, R.S. et al. *Nucleic Acids Research* **1989**, 17, 7253; Furdon, P.J. et al. *Nucleic Acids Research* **1989**, 17, 9193. Similar studies have been reported using *in vitro* mammalian RNase H cleavage assays. Agrawal, S. et al., *Proc. Natl. Acad. Sci. USA* **1990**, 87, 1401. In this case, a series of backbone modifications, including methylphosphonates, containing different

phosphodiester lengths were examined for cleavage efficiency. The minimum phosphodiester length required for efficient RNase H cleavage directed by oligonucleotides of this nature is five linkages. More recently, it has been shown that methylphosphonate/ phosphodiester chimeras display increased specificity and efficiency for target RNA cleavage using *E. coli* RNase H *in vitro*. Giles, R.V. et al., *Anti-Cancer Drug Design* **1992**, 7, 37. These compounds have also been reported to be effective antisense inhibitors in *Xenopus* oocytes and in cultured mammalian cells. Dagle, J.M. et al., *Nucleic Acids Res.* **1990**, 18, 4751; Potts, J.D., et al., *Proc. Natl. Acad. Sci. USA* **1991**, 88, 1516.

PCT Publication WO 90/15065, Froehler et al., discloses chimeric oligonucleotides "capped" at the 3′ and/or the 5′ end by phosphoramidite, phosphorothioate or phosphorodithioate linkages in order to provide stability against exonucleases while permitting RNAse H activation. PCT Publication WO 91/12323, Pederson et al., discloses chimeric oligonucleotides in which two regions with modified backbones (methyl phosphonates, phosphoromorpholidates, phosphoropiperazidates or phosphoramidates) which do not activate RNAse H flank a central deoxynucleotide region which does activate RNAse H cleavage. 2′-deoxy oligonucleotides have been stabilized against nuclease degradation while still providing for RNase H activation by positioning a short section of phosphodiester linked nucleotides between sections of backbone-modified oligonucleotides having phosphoramidate, alkylphosphonate or phosphotriester linkages. Dagle, J.M, Walder, J.A. and Weeks, D.L., *Nucleic Acids Research* **1990**, 18, 4751; Dagle, J.M., Weeks, D.L. and Walder, J.A., *Antisense Research And Development* **1991**, 1, 11; and Dagle, J.M., Andracki, M.E., DeVine, R.J. and Walder, J.A., *Nucleic Acids Research* **1991**, 19, 1805. While the phosphoramidate containing oligonucleotides were stabilized against exonucleases, each phosphoramidate linkage resulted in a loss of 1.6°C in the measured T_m value of the phosphoramidate containing oligonucleotides. Dagle, J.M., Andracki, M.E., DeVine, R.J. and

Walder, J.A., *Nucleic Acids Research* **1991**, 19, 1805. Such loss of the T_m value is indicative of a decrease in the hybridization between the oligonucleotide and its target strand.

Saison-Behmoaras, T., Tocque, B. Rey, I., Chassignol, M., Thuong, N.T. and Helene, C., *EMBO Journal* **1991**, 10, 1111, observed that even though an oligonucleotide was a substrate for RNase H, cleavage efficiency by RNase H was low because of weak hybridization to the mRNA.

Chimeric oligonucleotides are not limited to backbone modifications, though chimeric oligonucleotides containing 2′ ribose modifications mixed with RNase H-sensitive deoxy residues have not been as well characterized as the backbone chimeras. EP Publication 260,032 (Inoue et al.) and Ohtsuka et al., *FEBS Lett.* **1987**, 215, 327-330, employed 2′-O-methyl oligonucleotides (which alone would not be substrates for RNAse H) containing unmodified deoxy gaps to direct cleavage *in vitro* by *E. coli* RNase H to specific sites within the complementary RNA strand. These compounds required a minimum deoxy gap of four bases for efficient target RNA cleavage. However, oligonucleotides of this nature were not examined for cleavage efficiency using mammalian RNase H nor tested for antisense activity in cells. These oligonucleotides were not stabilized against nucleases.

Studies on the ability to direct RNase H cleavage and antisense activity of 2′ ribose modifications other than O-methyl have been extremely limited. Schmidt, S. et al., *Biochim. Biophys. Acta* **1992**, 1130, 41.

While it has been recognized that cleavage of a target RNA strand using an antisense oligonucleotide and RNase H would be useful, nuclease resistance of the oligonucleotide and fidelity of the hybridization are also of great importance. There has been a long-felt need for methods or materials that could both activate RNase H while concurrently maintaining or improving hybridization properties and providing nuclease resistance. There remains a long-felt need for such methods and materials for enhancing antisense activity.

OBJECTS OF THE INVENTION

It is an object of the invention to provide oligonucleotides complementary to ras mRNA which inhibit expression of the ras gene.

It is another object of the invention to provide oligonucleotides complementary to ras mRNA which specifically inhibit expression of an activated (mutant) form of the ras gene.

Yet another object of the invention is to provide stabilized oligonucleotides which inhibit expression of the ras gene.

Another object of the invention is to provide stabilized oligonucleotides complementary to ras mRNA and modified to increase their affinity for the ras mRNA target, which inhibit expression of the ras gene.

Still another object is to provide oligonucleotides which are complementary to ras mRNA and which are substrates for RNAse H.

An additional object of the invention is to provide oligonucleotides which inhibit proliferation of cancer cells. Methods of inhibiting proliferation of cancer cells are also an object of this invention.

Detection of the mutation from the normal (wild-type) to activated form of the ras gene is another object of the invention.

Differential diagnosis of morphologically similar tumors and identification of high-risk conditions based on the presence of the activated ras gene is yet another object of this invention.

A further object of this invention is to provide methods of diagnosis and treatment of conditions arising due to mutation of the gene from the wild-type to the mutant, activated form of the ras gene.

SUMMARY OF THE INVENTION

In accordance with the present invention oligonucleotides having modified internucleosidic linkages or

modified nucleosidic units are provided that are complementary to DNA or RNA. In one preferred embodiment, oligonucleotides that are complementary to DNA or RNA deriving from the human H-ras gene are provided. In certain preferred oligonucleotides of the invention are oligonucleotides having alternating linkages or chimeric oligonucleotides having alternating linkages.

It is preferred that these oligonucleotides be complementary to the translation initiation codon of the gene, and preferably that the oligonucleotides comprise a sequence CAT. In accordance with another preferred embodiment, oligonucleotides that are complementary to codon 12 of the activated H-ras gene are provided, preferably comprising a sequence GAC. In another such embodiment, oligonucleotides are provided that are complementary to and hybridize preferentially with the mutated codon 12 of the activated H-ras gene. In this embodiment, such oligonucleotide preferably comprises a sequence GAC.

Oligonucleotides of the invention are conveniently and desirably presented in a pharmaceutically acceptable carrier.

One preferred group of oligonucleotides of the inventions have at least one methylene(methylimino) linkage. A further preferred group oligonucleotides of the invention have at least one methylene(methylimino) linkage alternating with a phosphorothioate or phosphodiester linkage. An even further group of oligonucleotides of the invention are chimeric oligonucleotides having from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA and containing a first region having at least one methylene(methylimino) linkage and a second region having at least one phosphorothioate linkage. In a more preferred group of these chimeric oligonucleotides the second region is flanked by two of the first regions each of which includes at least one methylene(methylimino) linkage.

In a further preferred groups of chimeric oligonucleotide of the invention the first region includes at

least one nucleosidic unit modified at the 2′ position. Preferred for the 2 modifications are 2′-O-alkyl, 2′-O-substituted alkyl or 2′-fluoro modifications. Most preferred is 2′-O-methoxyethyl modifications. In the chimeric oligonucleotides of the invention, preferred oligonucleotides include the second region comprising 2′-deoxynucleotides. A first preferred length for this second region is at least four nucleotides long. A more preferred length for this second region is from five to nine nucleotides long.

In certain preferred chimeric oligonucleotides of the invention the first region includes at least one methylene(methylimino) linkage alternating with a phosphorothioate or phosphodiester linkage. In further preferred oligonucleotides of the invention the first region includes one, two or three methylene(methylimino) linkages alternating with phosphorothioate or phosphodiester linkages.

In accordance with other preferred embodiments, oligonucleotides complementary to ras mRNA are provided which inhibit ras expression and which, at once, have increased resistance to nucleases, have increased binding affinity for the ras mRNA target, and are substrates for RNAse H.

It is presently preferred that increased binding affinity is conveyed by modification of at least one nucleotide at the 2′ position of the sugar, most preferably comprising a 2′-O-alkyl, 2′-O-alkylamino, 2′-O-alkyl-O-alkyl or 2′-fluoro modification.

In some preferred embodiments, the oligonucleotides of the invention are chimeric oligonucleotides comprising at least one region which is modified to increase binding affinity for the complementary ras mRNA, and a region which is a substrate for RNAse H cleavage. In one such embodiment an RNAse H substrate region is flanked by two regions having increased ras mRNA binding affinity.

Other aspects of the invention are directed to methods for modulating the expression of the human ras gene in cells or tissues and for specifically modulating the expression

of the activated ras gene in cells or tissues suspected of harboring a mutation leading to such activation.

Some embodiments of the invention are directed to methods for inhibiting the expression of the ras gene and for specifically inhibiting the expression of the activated ras gene.

Additional aspects of the invention are directed to methods of detection of the ras gene in cells or tissues and specific detection of the activated ras gene in cells or tissues suspected of harboring said mutated gene. Such methods comprise contacting cells or tissues suspected of containing the human ras gene with oligonucleotides in accordance with the invention in order to detect said gene.

Other aspects of the invention are directed to methods for diagnostics and therapeutics of animals suspected of having a mutation leading to activation of the ras gene. Such methods comprise contacting the animal or cells or tissues or a bodily fluid from the animal with oligonucleotides in accordance with the invention in order to inhibit the expression of this gene, to treat conditions arising from activation of this gene, or to effect a diagnosis thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing dose-response inhibition of ras-luciferase fusion protein expression using oligonucleotides targeted to the H-ras translation initiation codon (AUG). Expression is measured by measurement of luciferase activity as assayed by amount of light emitted when luciferin is added.

Figure 2 is a bar graph showing dose-response inhibition of ras-luciferase fusion protein expression using oligonucleotides targeted to the mutated codon-12 region in activated H-ras. Expression is measured by measurement of luciferase activity as assayed by amount of light emitted when luciferin is added.

Figure 3 is a bar graph showing single-dose inhibition of ras-luciferase fusion protein expression by

antisense phosphorothioate compounds. Expression is measured by measurement of luciferase activity as assayed by amount of light emitted when luciferin is added.

Figure 4 is a table and bar graph summarizing data obtained for 13 antisense oligonucleotides specifically hybridizable with the activated H-ras gene. Shown for each oligonucleotide is its length, region of the activated ras gene to which it specifically hybridizes, and its activity in inhibiting expression of the ras-luciferase fusion protein.

Figure 5 shows the ras mRNA target sequence (shown 5' to 3') and locations and sequences of antisense oligonucleotides targeted to the H-ras translation initiation codon (AUG) and the codon 12 region. Antisense oligonucleotides are shown 3' to 5'. Figure 5A shows two 20-mers (2502 and 2503) targeted to the AUG and a series of oligonucleotides from 5 to 25 nucleotides in length, targeted to codon 12. Figure 5B shows oligonucleotides 2502, 2503, 6186 and 2570 in relation to the ras mRNA target sequence.

Figure 6 is a bar graph showing inhibition of ras-luciferase by various doses of oligonucleotides 2502, 2503, 6186 and uniformly 2'-O-methylated versions of these phosphorothioate oligonucleotides.

Figure 7 is a bar graph showing antisense inhibition of mutant (striped bars) and normal (solid bars) ras-luciferase by antisense oligonucleotides of various lengths.

Figure 8 is a series of 8 panels showing inhibition of ras in a dose-dependent manner. Solid lines are activity against wild-type, dotted lines show activity against activated ras.

Figure 9 is a two-part figure showing antisense oligonucleotide binding to the 47-mer H-ras RNA hairpin target. Figure 9A is a gel shift analysis of hairpin target with uniform 2'-O-methyl oligonucleotide (deoxy number = 0) and of hairpin target with a 2'-O-methyl chimeric oligonucleotide having a nine base deoxy gap (deoxy number = 9) as a function of oligonucleotide concentration. Lanes 1-8 contain the

following oligonucleotide concentrations: 1) none; 2) 10^{-11}M; 3) 10^{-10}M; 4) 10^{-9}M; 5) 10^{-8}M; 6) 10^{-7}M; 7) 10^{-6}M; 8) 10^{-5}M. Figure 9B is a graph showing fraction of hairpin target shifted vs. concentration of antisense oligonucleotide. ◇: Deoxy number= 17; ●: Deoxy number= 9; ▲: Deoxy number= 7; ○: Deoxy number= 5; △: Deoxy number= 3; ■: Deoxy number= 1; □: Deoxy number= 0. (Inset: structure of 47-mer H-ras hairpin target shown with sequence of oligonucleotide 2570).

Figure 10 is a gel showing RNAse H dependent cleavage of complementary H-ras RNA by 2'-O-methyl chimeric phosphorothioate oligonucleotides. Lane designations refer to the length of the centered deoxy gap.

Figure 11 is a two-part figure showing antisense activity of phosphorothioate 2'-O-methyl chimeric oligonucleotides targeted to ras codon-12 RNA sequences. Figure 11A is a bar graph showing single-dose activity (100 nM) of uniform 2'-O-methyl oligonucleotides, uniform deoxy oligonucleotides and chimeric 2'-O-methyl oligonucleotides containing centered 1-, 3-, 5-, 7- or 9-base deoxy gaps. Figure 11B is a line graph showing dose-response activity of uniform deoxy (▼) or 2'-O-methyl oligonucleotides containing centered 4-(■,◆), 5-(●), 7-(+) or 9-base (▲) deoxy gaps.

Figure 12 is a bar graph showing antisense activities of a uniform deoxy phosphorothioate and shortened chimeric oligonucleotides against ras-luciferase.

Figure 13 is a line graph showing correlation between antisense activity and ability to activate RNAse H as a function of deoxy gap length using phosphorothioate 2'-O-methyl oligonucleotides targeted against ras.

Figure 14 is a line graph showing dose response antisense activities of phosphorothioate 2'-modified chimeric oligonucleotides containing 7-base deoxy gaps. (▲), uniform deoxy phosphorothioate; (■), 2'-O-pentyl chimera; (●), 2'-O-propyl chimera; (◆), 2'-O-methyl chimera; (▼), 2'-fluoro chimera.

Figure 15 is a bar graph showing dose-dependent oligonucleotide inhibition of ras-luciferase by chimeric

oligonucleotides having various combinations of phosphorothioate and phosphodiester backbones and 2'-O-methyl and 2'-deoxy nucleotides.

Figure 16 is a line graph showing anti-tumor activity of ISIS 2503 against A549 human cell tumors in nude mice.

Figure 17 is a line graph showing anti-tumor activity of ras oligo ISIS 2503, administered with cationic lipid, against A549 human cell tumors in nude mice.

Figure 18 is a bar graph showing activity against Ha-ras of oligonucleotides with various 2' sugar modifications and phosphodiester (P=O) backbones compared to a 2'deoxyoligonucleotide with phosphorothioate (P=S) backbone.

Figure 19 is a bar graph showing antisense inhibition of Ki-ras mRNA expression in three human colon carcinoma cell lines, Calu1, SW480 and SW620.

Figure 20 is a bar graph showing inhibition of SW480 human carcinoma cell line proliferation by Ki-ras specific oligonucleotides ISIS 6957 and ISIS 6958.

Figure 21 is a bar graph showing selective inhibition of Ki-ras mRNA expression in human carcinoma SW480 cells, which express mutant Ki-ras, compared to HeLa cells, which express wild-type Ki-ras, when treated with oligonucleotide targeted to the mutant Ki-ras codon-12.

Figure 22 illustrates preferred chemical structures of certain oligonucleotides of the invention.

Figures 23 and 24 each show a table and a bar graph summarizing properties and inhibition responses for certain preferred antisense oligonucleotides specifically hybridizable with the activated H-ras gene or scrambled controls. The tables show the nucleobase composition and chemistry at each position along the length of the oligonucleotides. The bar graphs summarize the dose response inhibition of H-ras mRNA in T-24 cells for each of the tested doses of the oligonucleotides.

DETAILED DESCRIPTION OF THE INVENTION

Malignant tumors develop through a series of stepwise, progressive changes that lead to the loss of growth

control characteristic of cancer cells, i.e., continuous unregulated proliferation, the ability to invade surrounding tissues, and the ability to metastasize to different organ sites. Carefully controlled *in vitro* studies have helped define the factors that characterize the growth of normal and neoplastic cells and have led to the identification of specific proteins that control cell growth and differentiation. In addition, the ability to study cell transformation in carefully controlled, quantitative *in vitro* assays has led to the identification of specific genes capable of inducing the transformed cell phenotype. Such cancer-causing genes, or oncogenes, are believed to acquire transformation-inducing properties through mutations leading to changes in the regulation of expression of their protein products. In some cases such changes occur in non-coding DNA regulatory domains, such as promoters and enhancers, leading to alterations in the transcriptional activity of oncogenes, resulting in over- or under-expression of their gene products. In other cases, gene mutations occur within the coding regions of oncogenes, leading to the production of altered gene products that are inactive, overactive, or exhibit an activity that is different from the normal (wild-type) gene product.

To date, more than 30 cellular oncogene families have been identified. These genes can be categorized on the basis of both their subcellular location and the putative mechanism of action of their protein products. The ras oncogenes are members of a gene family which encode related proteins that are localized to the inner face of the plasma membrane. ras proteins have been shown to be highly conserved at the amino acid level, to bind GTP with high affinity and specificity, and to possess GTPase activity. Although the cellular function of ras gene products is unknown, their biochemical properties, along with their significant sequence homology with a class of signal-transducing proteins known as GTP binding proteins, or G proteins, suggest that ras gene products play a fundamental role in basic cellular regulatory functions relating to the transduction of extracellular signals across plasma membranes.

Three ras genes, designated H-ras, K-ras, and N-ras, have been identified in the mammalian genome. Mammalian ras genes acquire transformation-inducing properties by single point mutations within their coding sequences. Mutations in naturally occurring ras oncogenes have been localized to codons 12, 13, and 61. The sequences of H-ras, K-ras and N-ras are known. Capon et al., *Nature 302* **1983**, 33-37; Kahn et al., *Anticancer Res.* **1987**, 7, 639-652; Hall and Brown, *Nucleic Acids Res.* **1985**, 13, 5255-5268. The most commonly detected activating ras mutation found in human tumors is in codon 12 of the H-ras gene in which a base change from GGC to GTC results in a glycine-to-valine substitution in the GTPase regulatory domain of the ras protein product. Tabin, C.J. et al., *Nature* **1982**, 300, 143-149; Reddy, P.E. et al., *Nature* **1982**, 300, 149-152; Taparowsky, E. et al., *Nature* **1982**, 300, 762-765. This single amino acid change is thought to abolish normal control of ras protein function, thereby converting a normally regulated cell protein to one that is continuously active. It is believed that such deregulation of normal ras protein function is responsible for the transformation from normal to malignant growth.

The present invention provides oligonucleotides for inhibition of human ras gene expression. Such oligonucleotides specifically hybridize with selected DNA or mRNA deriving from a human ras gene. The invention also provides oligonucleotides for selective inhibition of expression of the mutant form of ras.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages as well as oligomers having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

Specific examples of preferred oligonucleotides of the invention contain phosphodiester,, phosphorothioates or heteroatom intersugar linkages or chimeric and/or alternating mixtures of these linkages. Preferred heteroatom intersugar linkage are those with CH_2-NH-O-CH_2, CH_2-N(CH_3)-O-CH_2, CH_2-O-N(CH_3)-CH_2, CH_2-N(CH_3)-N(CH_3)-CH_2 and O-N(CH_3)-CH_2-CH_2 backbones (where phosphodiester is O-P(=O)-O-CH_2). Most preferred is the CH_2-N(CH_3)-O-CH_2 linkage. The CH_2-N(CH_3)-O-CH_2 linkage can be named in various ways including as 3'-de(oxyphosphinico)-3'-[methylene(methylimino)] linkage that can be shorten to a methylene(methylimino) linkage or shorten still to the acronym "MMI" linkage. In certain chimeric mixed backbones of the invention regions of phosphorothioate nucleotides are flanked by regions of heteroatom linkages or by regions of alternating heteroatom and one of phosphodiester or phosphorothioate linkages.

Other preferred oligonucleotides may contain alkyl and halogen-substituted sugar moieties. Preferred substituted sugar moieties comprise one of the following at the 2' position: F, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$ or $O(CH_2)_n$-O-$(CH_2)_nCH_3$ where n is from 0 to about 10.

The oligonucleotides of the invention include the standard nucleosidic bases including but not limited to purin-9-yl and pyrimid-1-yl heterocycles including adenine, guanine, thymine, uracil and cytosine. Other preferred embodiments may include at least one modified base form. Some specific examples of such modified bases include 2-(amino)adenine, 2-(methylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalklyamino)adenine, 7-deaza-propynyl adenine or guanine, 7-deaza-8-aza adenine or guanine, 5-methylcytosine, 5-propynyl uracil, 2-thiouracil, 2-aminouracil, and psuedouracil.

Preferred oligonucleotides of this invention may, at once, comprise nucleotides modified to increase their resistance to nucleases, comprise nucleotides modified to increase their affinity for ras mRNA, and comprise nucleotides which are substrates for RNAse H. In one preferred embodiment, a chimeric oligonucleotide comprises at least one region

modified to increase ras mRNA binding affinity, and a region which is a substrate for RNAse H. The oligonucleotide is also modified to enhance nuclease resistance. In a more preferred embodiment, the region which is a substrate for RNAse H is flanked by two regions which are modified to increase ras mRNA binding affinity. The effect of such modifications is to greatly enhance antisense oligonucleotide inhibition of ras gene expression.

The oligonucleotides in accordance with this invention preferably comprise from about 8 to about 50 nucleic acid base units. It is more preferred that such oligonucleotides comprise from about 8 to 30 nucleic acid base units, and still more preferred to have from about 13 to 25 nucleic acid base units. As will be appreciated, a nucleic acid base unit is a base-sugar combination suitably bound to adjacent nucleic acid base unit through phosphodiester or other bonds.

"Hybridization," in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them.

"Specifically hybridizable" indicates a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable.

Antisense Oligonucleotide Inhibition of ras-Luciferase Gene Expression: A series of antisense phosphorothioate oligonucleotides targeted to either the H-ras translation initiation codon or the codon-12 point mutation of activated H-ras were screened using the ras-luciferase reporter gene system described in Examples 2-5. Of this initial series, six oligonucleotides were identified that gave significant and reproducible inhibition of ras-luciferase activity. The base sequences, sequence reference numbers and SEQ ID numbers of

these oligonucleotides (all are phosphorothioates) are shown in Table 1.

TABLE 1

OLIGO REF NO	SEQUENCE	SEQ ID NO:
2502	CTT-ATA-TTC-CGT-CAT-CGC-TC	1
2503	TCC-GTC-ATC-GCT-CCT-CAG-GG	2
2570	CCA-CAC-CGA-CGG-CGC-CC	3
2571	CCC-ACA-CCG-ACG-GCG-CCC-A	4
2566	GCC-CAC-ACC-GAC-GGC-GCC-CAC	5
2560	TGC-CCA-CAC-CGA-CGG-CGC-CCA-CC	6

Figure 1 shows a dose-response experiment in which cells expressing either the normal ras-luciferase reporter gene or the mutant ras-luciferase reporter gene were treated with increasing concentrations of the phosphorothioate oligonucleotide 2503 (SEQ ID NO: 2). This compound is targeted to the translational initiation codon of H-ras RNA transcripts. As shown in Figure 1, treatment of cells with this oligonucleotide resulted in a dose-dependent inhibition of ras-luciferase activity, displaying IC50 values of approximately 50 nM for both the normal and the mutant ras targets. The control oligonucleotide is a random phosphorothioate oligonucleotide, 20 bases long. Results are expressed as percentage of luciferase activity in transfected cells not treated with oligonucleotide. The observation that an oligonucleotide targeted to the ras translation initiation codon is equally effective in reducing both mutant and normal ras expression is expected since the two targets have identical sequence compositions in the region surrounding the AUG translation initiation site.

Figure 2 shows a dose-response experiment in which cells were treated with phosphorothioate oligonucleotide 2570 (SEQ ID NO: 3), a compound that is targeted to the codon-12 point mutation of mutant (activated) H-ras RNA. The control oligonucleotide is a random phosphorothioate oligonucleotide, 20 bases long. Results are expressed as percentage of

luciferase activity in transfected cells not treated with oligonucleotide. As the figure shows, treatment of cells with increasing concentrations of this oligonucleotide resulted in a dose-dependent inhibition of ras-luciferase activity in cells expressing either the mutant form or the normal form of ras-luciferase. However, careful examination of the data shows that at low concentrations, oligonucleotide 2570 displayed approximately threefold selectivity toward the mutant form of ras-luciferase as compared to the normal form. In fact, 2570 displayed an IC50 value for the mutant form of ras-luciferase of approximately 100 nM whereas the same compound displayed in IC50 value of approximately 250 nM for the unmutated form.

Figure 3 shows the results of a typical experiment in which cells expressing either the normal form or the mutant form of ras-luciferase were treated with a single dose (0.5 μM) of oligonucleotide targeted to either the translation initiation codon of H-ras or the codon-12 point mutation. The antisense phosphorothioate oligonucleotides tested are shown in Table 1. The control oligonucleotide (2504) is a random phosphorothioate oligonucleotide, 20 bases long. Results are expressed as percentage of luciferase activity in transfected cells not treated with oligonucleotide. As shown in Figure 3, compound 2503 (SEQ ID NO: 2), targeted to the ras translational initiation codon, was most effective in inhibiting ras-luciferase activity. Of the three compounds targeted to the codon-12 point mutation of activated H-ras, only the 17-mer oligonucleotide 2570 (SEQ ID NO: 3) displayed selectivity toward the mutated form of ras-luciferase as compared to the normal form. This is also shown in Figure 4, which summarizes data obtained with all 13 antisense oligonucleotides complementary to the activated H-ras gene, as well as a scrambled control oligonucleotide (1966) and a control oligonucleotide (2907) complementary to the codon-12 region of wild-type ras. Shown for each oligonucleotide is its length, region to which it is complementary, and its activity in suppressing expression of the ras-luciferase fusion protein. The longer phosphorothioates targeted to the codon-12 point

mutation, while displaying substantial antisense activity toward ras-luciferase expression, did not demonstrate selective inhibition of expression of the mutant form of ras-luciferase. Phosphorothioate oligonucleotides targeted to the codon-12 point mutation that were less than 17 nucleotides in length did not show activity to either form of ras-luciferase. These results demonstrate effective antisense activity of phosphorothioate oligonucleotides targeted to ras sequences.

Antisense oligonucleotides specifically hybridizable with the H-ras AUG: Three 20-base phosphorothioate oligonucleotides, targeted to the H-ras AUG codon, were compared for their ability to inhibit ras-luciferase expression in transient transfection assays as described in Examples 2-5. Results are shown in Figures 5A and 5B. These oligonucleotides, ISIS 2502 (SEQ ID NO: 1), 2503 (SEQ ID NO: 2) and 6186 (SEQ ID NO: 7) shown in Table 2, were tested for inhibition of ras-luciferase expression at a single dose (100 nM) in HeLa cells. All three AUG-targeted oligonucleotides were effective in inhibiting ras-luciferase expression. These three phosphorothioate oligonucleotides were also prepared with a 2'-O-methyl modification on each sugar. The 2'-O-methylated version of ISIS 2503 (SEQ ID NO: 2) also inhibited ras-luciferase expression. This is shown in Figure 6.

TABLE 2

Antisense oligonucleotides targeted to mutant H-ras (Oligonucleotide sequences shown 5' to 3')

OLIGO	LENGTH	TARGET	SEQUENCE	SEQ. ID NO.
2502	20	AUG	CTTATATTCCGTCATCGCTC	1
2503	20	AUG	TCCGTCATCGCTCCTCAGGG	2
6186	20	AUG	TATTCCGTCATCGCTCCTCA	7
2563	5	CODON 12	CGACG	8
2564	7	CODON 12	CCGACGG	9
2565	9	CODON 12	ACCGACGGC	10
2567	11	CODON 12	CACCGACGGCG	11
2568	13	CODON 12	ACACCGACGGCGC	12
2569	15	CODON 12	CACACCGACGGCGCC	13
3426	16	CODON 12	CCACACCGACGGCGCC	14
3427	16	CODON 12	CACACCGACGGCGCCC	15
2570	17	CODON 12	CCACACCGACGGCGCCC	3
3428	18	CODON 12	CCCACACCGACGGCGCCC	16
3429	18	CODON 12	CCACACCGACGGCGCCCA	17
2571	19	CODON 12	CCCACACCGACGGCGCCCA	4
2566	21	CODON 12	GCCCACACCGACGGCGCCCAC	5
2560	23	CODON 12	TGCCCACACCGACGGCGCCCACC	6
2561	25	CODON 12	TTGCCCACACCGACGGCGCCCACCA	18
907	17	CODON 12 (wild type)	CCACACCGCCGGCGCCC	19

Oligonucleotide length affects antisense activity and specificity: Oligonucleotides targeted to the H-ras codon- 12 point mutation also were effective in inhibiting expression of ras-luciferase. A series of eleven phosphorothioate oligonucleotides, ranging in length between 5 and 25 bases, were made and tested for ability to inhibit mutant and wild type ras-luciferase in transient transfection assays as described in Examples 2-5. The oligonucleotides are shown in Table 2. At 100 nM oligonucleotide concentration, oligonucleotides 15 bases or greater in length were found to

inhibit expression of the mutant H-ras target. Selective inhibition of mutant over wild type ras-luciferase expression was observed for oligonucleotides between 15 and 19 bases in length. The maximum selectivity observed for inhibition of mutant ras-luciferase relative to wild type was for the 17-mer 2570 (SEQ ID NO: 3) and was approximately 4-fold. In order to demonstrate that 2570 was acting in a sequence-specific manner, a variant of this compound was tested (2907; SEQ ID NO: 19) in which the central adenosine residue was replaced with cytosine, making this oligonucleotide perfectly complementary to the wild type H-ras target. Hence, this oligonucleotide will contain a single mismatch at the center of the oligonucleotide/RNA duplex when fully hybridized to the mutant H-ras sequence. As shown in Figure 7, oligonucleotide 2907 selectively inhibited expression of wild type ras-luciferase relative to mutant ras-luciferase, with the difference being approximately 5-fold at an oligonucleotide dosage of 100 nM.

Two 16-mers and two 18-mers complementary to the mutant codon-12 region (Figure 5 and Table 2) were tested as described in Examples 2-5. Figure 8 shows the results of an experiment in which antisense activity and mutant selectivity was determined for oligonucleotides of length 13, 15, 16, 17, 18 and 19 bases in a dose-dependent manner. The results obtained with these oligonucleotides demonstrated that the compounds that were active against mutant H-ras sequences also showed selectivity; oligonucleotides of length 16 (SEQ ID NO: 14 and SEQ ID NO: 15) and 17 bases (SEQ ID NO: 3) displayed the greatest selectivity (4- and 5-fold, respectively). The 13 base compound, 2568 (SEQ ID NO: 12), did not display antisense activity at any of the tested concentrations.

Chimeric 2'-O-methyl oligonucleotides with deoxy gaps: Based on the sequence of the mutant-selective 17-mer (2570), a series of chimeric phosphorothioate 2'-O-methyl oligonucleotides were synthesized in which the end regions consisted of 2'-O-methyl nucleosides and the central residues formed a "deoxy gap". The number of deoxy residues ranged from

zero (full 2'-O-methyl) to 17 (full deoxy). These oligonucleotides are shown in Table 3.

TABLE 3

Chimeric phosphorothioate oligonucleotides having 2'-O-methyl ends (bold) and central deoxy gap (Mutant codon-12 target)

OLIGO #	DEOXY	SEQUENCE	SEQ ID NO
4122	0	CCACACCGACGGCGCCC	3
3975	1	CCACACCGACGGCGCCC	3
3979	3	CCACACCGACGGCGCCC	3
4236	4	CCACACCGACGGCGCCC	3
4242	4	CCACACCGACGGCGCCC	3
3980	5	CCACACCGACGGCGCCC	3
3985	7	CCACACCGACGGCGCCC	3
3984	9	CCACACCGACGGCGCCC	3
2570	17	CCACACCGACGGCGCCC	3

These oligonucleotides were characterized for hybridization efficiency as described in Example 6, ability to direct RNase H cleavage *in vitro* using mammalian RNase H as described in Example 8, and for antisense activity. Antisense activity against full length H-ras mRNA was determined using a transient co-transfection reporter gene system in which H-ras gene expression was monitored using a ras-responsive enhancer element linked to the reporter gene luciferase, as described in Example 9.

Hybridization of phosphorothioate antisense oligonucleotides to single stranded 25-mer RNA targets: Figure 5 and Table 2 show the sequences of 15 phosphorothioate oligonucleotides targeted to activated H-ras mRNA containing the codon 12 G→U point mutation. These oligonucleotides range between 5 and 25 bases in length and are centered around the point mutation. Melting temperatures (T_m) for these antisense phosphorothioates against mutant and wild type 25-mer RNA targets at 4 μM oligonucleotide concentration were measured. T_m increased with increasing chain length and, for any chain

length, T_m for hybridization to the mutant target was greater than that for the wild type target. Oligonucleotide 2907 is a phosphorothioate 17-mer variant of 2570 in which the central adenosine residue was replaced with cytosine, making this oligonucleotide perfectly complementary to the wild type H-ras target. As expected, the melting temperature for hybridization of this oligonucleotide to the wild type target was greater than that for the mutant target, which now contains a single mismatch in the oligonucleotide/RNA duplex at the site of the point mutation. For the 17-mer phosphorothioate (2570) that is perfectly complementary to the mutant H-ras target, thermodynamic parameters were also obtained from dependence of T_m on oligonucleotide concentration. These data were used to determine the free energy difference ($\Delta\Delta G^\circ_{37}$) between hybridization of oligonucleotides to the mutant target and to the wild type target. For a given oligonucleotide, $\Delta\Delta G^\circ_{37}$ can be obtained from T_m dependence on oligonucleotide concentration. Borer, P.N. et al., *J. Mol. Biol.* **1974**, 86, 843-853. The $\Delta\Delta G^\circ_{37}$ for 2570 was calculated to be +1.8 kcal/mole.

The maximum degree of selectivity that can be achieved for targeting mutant over wild type ras increases significantly as $\Delta\Delta G^\circ_{37}$ increases. Therefore, chemical modifications of the antisense oligonucleotide which increase $\Delta\Delta G^\circ_{37}$ enhance selectivity. One such modification is 2,6-diaminopurine, which is believed to bind more tightly than dA to U and less tightly than dA to G, and thus to increase $\Delta\Delta G^\circ_{37}$ for the A-U -->A-G mismatch. The substrate requirements of RNase H can also be exploited to obtain selectivity according to the teachings of this invention. If the enzyme is unable to bind or cleave a mismatch, additional selectivity will be obtained beyond that conferred by $\Delta\Delta G^\circ_{37}$ by employing chimeric oligonucleotides that place the RNAse H recognition site at the mismatch. This has been found to be the case; RNase H can indeed discriminate between a fully matched duplex and one containing a single mismatch.

Hybridization of "deoxy gap" oligonucleotides to short oligonucleotide targets: Hybridization analysis of the 2′-O-methyl deoxy gap series against a 25-mer synthetic oligoribonucleotide complement as described in Example 6 demonstrated that T_m values for a given oligonucleotide correlated directly with 2′-O-methyl content. As 2′-O-methyl modifications were replaced with deoxy substituents, T_m values were reduced at approximately 1.5°C per modification. In these experiments, the T_m values of the oligonucleotides containing 2′-O-methyl modifications were higher than the T_m values of the full deoxy compound of the same sequence.

Hybridization of "deoxy gap" oligonucleotides to a structured RNA target: In further experiments oligonucleotides were hybridized to a larger H-ras target which contains a stable stem loop structure in the codon 12 region. Effects of 2′-O-methyl modifications on antisense hybridization to the structured H-ras target were determined by gel shift analysis as described in Example 7.

As shown in Figure 9, the full deoxy 17-mer formed the least stable duplex with the hairpin target; the full 2′-O-methyl 17-mer formed the most stable duplex. As deoxy gap size was decreased in these oligonucleotides, increasing the number of 2′-O-methyl residues increased duplex stability.

Secondary and tertiary structure of the RNA target affects hybridization of antisense oligonucleotides. A series of 11-mer chimeric oligonucleotides were made which hybridize to various regions of the ras hairpin target. ISIS 5055 hybridizes to the left side of the stem region (as the hairpin is displayed in Figure 9). ISIS 5056 hybridizes to the left side of the loop. ISIS 5091 hybridizes to the right side of the loop and ISIS 5147 hybridizes to the right side of the stem. All are uniform phosphorothioates with centered 5-deoxy gaps flanked by 2′-O-methyl regions. Only the 11-mer targeted to the left side of the loop bound measurably to the target. The other 11-mers did not bind measurably. Longer versions of these oligonucleotides were also made; these 13-mer oligoribonucleotides all demonstrated measurable binding to the

hairpin target, with the oligonucleotide targeted to the left side of the loop demonstrating the tightest binding in the gel-shift assay.

RNAse H cleavage directed by deoxy gapped oligonucleotides: Ability of 2'-O-methyl deoxy gap oligonucleotides to direct RNase H cleavage of a complementary RNA was determined *in vitro* using HeLa nuclear extracts as a source of RNase H as described in Example 8. As shown in Figure 10, no cleavage was observed with the fully modified 2'-O-methyl oligonucleotide or one containing a single deoxy residue. Oligonucleotides with a deoxy length of three, four, five, seven or nine were able to direct RNase H cleavage. Deoxy gaps of five, seven or nine are preferred and gaps of seven or nine are most preferred.

Antisense activity of deoxy-gapped oligonucleotides against full length ras mRNA: The beneficial properties of enhanced target affinity conferred by 2'-O-methyl modifications can be exploited for antisense inhibition provided these compounds are equipped with RNase H-sensitive deoxy gaps of the appropriate length. 2'-O-methyl deoxy gap oligonucleotides were tested for antisense activity against the full length H-ras mRNA using the H-ras transactivation reporter gene system described in Example 9. Antisense experiments were performed initially at a single oligonucleotide concentration (100 nM). As shown in Figure 11, chimeric 2'-O-methyl oligonucleotides containing deoxy gaps of five or more residues inhibited H-ras gene expression. These compounds displayed activities greater than that of the full deoxy parent compound.

Dose response experiments were performed using these active compounds, along with the 2'-O-methyl chimeras containing four deoxy residues. As shown in Figure 11B, oligonucleotide-mediated inhibition of full-length H-ras by these oligonucleotides was dose-dependent. The most active compound was the seven-residue deoxy chimera, which displayed an activity approximately five times greater than that of the full deoxy oligonucleotide.

Shortened chimeric oligonucleotides: Enhanced target affinity conferred by the 2'-O-methyl modifications was found to confer activity on short chimeric oligonucleotides. A series of short 2'-O-methyl chimeric oligonucleotides were tested for T_m and antisense activity vs. full length ras as described in Example 9. Table 4 shows T_ms for oligonucleotides 11, 13, 15 and 17 nucleotides in length, having deoxy gaps either 5 bases long or 7 bases long. In sharp contrast to the full deoxy 13-mer, both 2'-O-methyl chimeric 13-mers inhibited ras expression, and one of the 11-mers was also active. This is shown in Figure 12.

TABLE 4

LENGTH	T_m (°C)	SEQUENCE	SEQ ID NO:
17	77.2	CCACACCGACGGCGCCC	3
15	69.8	CACACCGACGGCGCC	13
13	62.1	ACACCGACGGCGC	12
11	47.3	CACCGACGGCG	11
17	74.6	CCACACCGACGGCGCCC	3
15	66.2	CACACCGACGGCGCC	13
13	58.0	ACACCGACGGCGC	12
11	27.7	CACCGACGGCG	11

Relative antisense activity and ability to activate RNase H cleavage *in vitro* by chimeric 2'-O-methyl oligonucleotides is well correlated with deoxy length (Figure 13).

Asymmetrical deoxy gaps: It is not necessary that the deoxy gap be in the center of the chimeric molecule. It was found that chimeric molecules having the nucleotides of the region at one end modified at the 2' position to enhance binding and the remainder of the molecule unmodified (2'deoxy) can still inhibit ras expression. Oligonucleotides of SEQ ID NO: 3 (17-mer complementary to mutant codon 12) in which a 7-deoxy gap was located at either the 5' or 3' side of the 17-mer, or at different sites within the middle of the molecule, all demonstrated RNase H activation and antisense activity. However, a 5-base gap was found to be more sensitive to

placement, as some gap positions rendered the duplex a poor activator of RNase H and a poor antisense inhibitor. Therefore, a 7-base deoxy gap is preferred.

Other sugar modifications: The effects of other 2′ sugar modifications besides 2′-O-methyl on antisense activity in chimeric oligonucleotides have been examined. These modifications are listed in Table 5, along with the T_m values obtained when 17-mer oligonucleotides having 2′-modified nucleotides flanking a 7-base deoxy gap were hybridized with a 25-mer oligoribonucleotide complement as described in Example 6. A relationship was observed for these oligonucleotides between alkyl length at the 2′ position and T_m. As alkyl length increased, T_m decreased. The 2′-fluoro chimeric oligonucleotide displayed the highest T_m of the series.

TABLE 5

Correlation of T_m with Antisense Activity
2′-modified 17-mer with 7-deoxy gap
CCACACCGACGGCGCCC (SEQ ID NO: 3)

2′ MODIFICATION	T_m (°C)	IC50 (nM)
Deoxy	64.2	150
O-Pentyl	68.5	150
O-Propyl	70.4	70
O-Methyl	74.7	20
Fluoro	76.9	10

These 2′ modified oligonucleotides were tested for antisense activity against H-ras using the transactivation reporter gene assay described in Example 9. As shown in Figure 14 and Table 5, all of these 2′ modified chimeric compounds inhibited ras expression, with the 2′-fluoro 7-deoxy-gap compound the most active. A 2′-fluoro chimeric oligonucleotide with a centered 5-deoxy gap was also active.

Chimeric phosphorothioate oligonucleotides having SEQ ID NO: 3 having 2′-O-propyl regions surrounding a 5-base or 7-base deoxy gap were compared to 2′-O-methyl chimeric oligonucleotides. ras expression in T24 cells was inhibited

by both 2'-O-methyl and 2'-O-propyl chimeric oligonucleotides with a 7-deoxy gap and a uniform phosphorothioate backbone. When the deoxy gap was decreased to five nucleotides, only the 2'-O-methyl oligonucleotide inhibited ras expression.

Antisense oligonucleotide inhibition of H-ras gene expression in cancer cells: Two phosphorothioate oligonucleotides (2502, 2503) complementary to the ras AUG region were tested as described in Example 10, along with chimeric oligonucleotides (4998, 5122) having the same sequence and 7-base deoxy gaps flanked by 2'-O-methyl regions. These chimeric oligonucleotides are shown in Table 6.

TABLE 6

Chimeric phosphorothioate oligonucleotides having 2'-O-methyl ends (bold) and central deoxy gap (AUG target)

OLIGO #	DEOXY	SEQUENCE	SEQ ID NO:
2502	20	CTTATATTCCGTCATCGCTC	1
4998	7	**CTTATA**TTCCGTC**ATCGCTC**	1
2503	20	TCCGTCATCGCTCCTCAGGG	2
5122	7	**TCCGTC**ATCGCTC**CTCAGGG**	2

Compound 2503 inhibited ras expression in T24 cells by 71%, and the chimeric compound (4998) inhibited ras mRNA even further (84% inhibition). Compound 2502, also complementary to the AUG region, decreased ras RNA levels by 26% and the chimeric version of this oligonucleotide (5122) demonstrated 15% inhibition. Also included in this assay were two oligonucleotides targeted to the mutant codon 12. Compound 2570 (SEQ ID NO: 3) decreased ras RNA by 82% and the 2'-O-methyl chimeric version of this oligonucleotide with a seven-deoxy gap (3985) decreased ras RNA by 95%.

Oligonucleotides 2570 and 2503 were also tested to determine their effects on ras expression in HeLa cells, which have a wild-type (i.e., not activated) H-ras codon 12. While both of these oligonucleotides inhibited ras expression in T24 cells (having activated codon 12), only the oligonucleotide (2503) specifically hybridizable with the ras AUG inhibited ras

expression in HeLa cells. Oligonucleotide 2570 (SEQ ID NO: 3), specifically hybridizable with the activated codon 12, did not inhibit ras expression in HeLa cells, because these cells lack the activated codon-12 target.

Oligonucleotide 2570, a 17-mer phosphorothioate oligonucleotide complementary to the codon 12 region of activated H-ras, was tested for inhibition of ras expression (as described in Example 10) in T24 cells along with chimeric phosphorothioate 2'-O-methyl oligonucleotides 3980, 3985 and 3984, which have the same sequence as 2570 and have deoxy gaps of 5, 7 and 9 bases, respectively (shown in Table 3). The fully 2'-deoxy oligonucleotide 2570 and the three chimeric oligonucleotides decreased ras mRNA levels in T24 cells. Compounds 3985 (7-deoxy gap) and 3984 (9-deoxy gap) decreased ras mRNA by 81%; compound 3980 (5-deoxy gap) decreased ras mRNA by 61%. Chimeric oligonucleotides having this sequence, but having 2'-fluoro-modified nucleotides flanking a 5-deoxy (4689) or 7-deoxy (4690) gap, inhibited ras mRNA expression in T24 cells, with the 7-deoxy gap being preferred (82% inhibition, vs 63% inhibition for the 2'-fluoro chimera with a 5-deoxy gap).

Antisense oligonucleotide inhibition of proliferation of cancer cells: Three 17-mer oligonucleotides having the same sequence (SEQ ID NO: 3), complementary to the codon 12 region of activated ras, were tested for effects on T24 cancer cell proliferation as described in Example 11. 3985 has a 7-deoxy gap flanked by 2'-O-methyl nucleotides, and 4690 has a 7-deoxy gap flanked by 2'-F nucleotides (all are phosphorothioates). Effects of these oligonucleotides on cancer cell proliferation correlated well with their effects on ras mRNA expression shown by Northern blot analysis: oligonucleotide 2570 inhibited cell proliferation by 61%, the 2'-O-methyl chimeric oligonucleotide 3985 inhibited cell proliferation by 82%, and the 2'-fluoro chimeric analog inhibited cell proliferation by 93%.

In dose-response studies of these oligonucleotides on cell proliferation, the inhibition was shown to be dose-dependent in the 25 nM-100 nM range. IC50 values of 44 nM, 61

nM and 98 nM could be assigned to oligonucleotides 4690, 3985 and 2570, respectively. The random oligonucleotide control had no effect at the doses tested.

The effect of ISIS 2570 on cell proliferation was cell type-specific. The inhibition of T24 cell proliferation by this oligonucleotide was four times as severe as the inhibition of HeLa cells by the same oligonucleotide (100 nM oligonucleotide concentration). ISIS 2570 is targeted to the activated (mutant) ras codon 12, which is present in T24 but lacking in HeLa cells, which have the wild-type codon 12.

Chimeric backbone-modified oligonucleotides: Oligonucleotides discussed in previous examples have had uniform phosphorothioate backbones. The 2'modified chimeric oligonucleotides discussed above are not active in uniform phosphodiester backbones. A chimeric oligonucleotide was synthesized (ISIS 4226) having 2'-O-methyl regions flanking a 5-nucleotide deoxy gap, with the gap region having a P=S backbone and the flanking regions having a P=O backbone. Another chimeric oligonucleotide (ISIS 4223) having a P=O backbone in the gap and P=S in flanking regions was also made. These oligonucleotides are shown in Table 7.

Additional oligonucleotides were synthesized, completely 2'deoxy and having phosphorothioate backbones containing either a single phosphodiester (ISIS 4248), two phosphodiesters (ISIS 4546), three phosphodiesters (ISIS 4551), four phosphodiesters (ISIS 4593), five phosphodiesters (ISIS 4606) or ten phosphodiester linkages (ISIS-4241) in the center of the molecule. These oligonucleotides are also shown in Table 7.

TABLE 7

Chimeric backbone (P=S/P=O) oligonucleotides having 2'-O-methyl ends (bold) and central deoxy gap (backbone linkages indicated by s (P=S) or o (P=O) Mutant codon-12 target

OLIGO #	P=S	SEQUENCE	SEQ ID NO:
2570	16	CsCsAsCsAsCsCsGsAsCsGsGsCsGsCsCsC	3
4226	5	CoCoAoCoAoCsCsGsAsCsGoGoCoGoCoCoC	3
4233	11	CsCsAsCsAsCoCoGoAoCoGsGsCsGsCsCsC	3
4248	15	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
4546	14	CsCsAsCsAsCsCsGoAoCsGsGsCsGsCsCsC	3
4551	13	CsCsAsCsAsCsCsGoAoCoGsGsCsGsCsCsC	3
4593	12	CsCsAsCsAsCsCoGoAoCoGsGsCsGsCsCsC	3
4606	11	CsCsAsCsAsCsCoGoAoCoGoGsCsGsCsCsC	3
4241	6	CsCsAsCoAoCoCoGoAoCoGoGoCoGsCsCsC	3

Oligonucleotides were incubated in crude HeLa cellular extracts at 37°C to determine their sensitivity to nuclease degradation as described in Dignam et al., *Nucleic Acids Res.* 1983, 11, 1475-1489. The oligonucleotide (4233) with a five-diester gap between phosphorothioate/2'-O-methyl wings had a $T_{1/2}$ of 7 hr. The oligonucleotide with a five-phosphorothioate gap in a phosphorothioate/2'-O-methyl molecule had a $T_{1/2}$ of 30 hours. In the set of oligonucleotides having one to ten diester linkages, the oligonucleotide (4248) with a single phosphodiester linkage was as stable to nucleases as was the full-phosphorothioate molecule, ISIS 2570, showing no degradation after 5 hours in HeLa cell extract. Oligonucleotides with two-, three and four-diester gaps had $T_{1/2}$ of approximately 5.5 hours, 3.75 hours, and 3.2 hours, and oligonucleotides with five or ten deoxy linkages had $T_{1/2}$ of 1.75 hours and 0.9 hours, respectively.

Antisense activity of chimeric backbone-modified oligonucleotides: A uniform phosphorothioate backbone is not required for antisense activity. ISIS 4226 and ISIS 4233 were tested in the ras-luciferase reporter system for effect on ras expression as described in Examples 2-5, along with ISIS 2570

(fully phosphorothioate/all deoxy), ISIS 3980 (fully phosphorothioate, 2'-O-methyl wings with deoxy gap) and ISIS 3961 (fully phosphodiester, 2'-O-methyl wings with deoxy gap). All of the oligonucleotides having a P=S (i.e., nuclease-resistant) gap region inhibited ras expression. This is shown in Figure 15. The two completely 2'deoxy oligonucleotides having phosphorothioate backbones containing either a single phosphodiester (ISIS 4248) or ten phosphodiester linkages (ISIS 4241) in the center of the molecule were also assayed for activity. The compound containing a single P=O was just as active as a full P=S molecule, while the same compound containing ten P=O was completely inactive.

Chimeric phosphorothioate oligonucleotides of SEQ ID NO: 3 were made, having a phosphorothioate backbone in the 7-base deoxy gap region only, and phosphodiester in the flanking regions, which were either 2'-O-methyl or 2'-O-propyl. The oligonucleotide with the 2'-O-propyl diester flanking regions was able to inhibit ras expression.

Inhibition of ras-luciferase gene expression by antisense oligonucleotides containing modified bases: A series of antisense phosphorothioate oligonucleotides complementary to the codon-12 point mutation of activated ras were synthesized as described, having a 2-(amino)adenine at the position complementary to the uracil of the mutated codon 12. Because the amino group at the 2-position of the adenine is able to hydrogen bond with the oxygen in the 2-position on the uracil, three hydrogen bonds instead of the usual two are formed. This serves to greatly stabilize the hybridization of the 2-(amino)adenine-modified antisense oligonucleotide to the activated ras gene, while destabilizing or having no net effect on the stability of this oligonucleotide to the wild-type codon 12, because of the modified A-G mismatch at this position. This increases the specificity of the modified oligonucleotide for the desired target.

An oligonucleotide having a single 2,6-(diamino)adenosine at this position in an otherwise unmodified uniform phosphorothioate 17-mer (sequence identical to 2570, SEQ ID NO:

3) was found to be at least as effective an RNase H substrate as the 2570 sequence. It is therefore expected to be an effective antisense molecule. An oligonucleotide having a single 2,-(diamino)adenosine at this position in a deoxy gapped phosphorothioate oligonucleotide of the same sequence also demonstrates RNase H activation.

***in vivo* anti-tumor data:** ISIS 2503 (SEQ ID NO: 2) has been evaluated for activity against human tumors *in vivo* as described in Examples 13 and 14. These studies employed a human lung adenocarcinoma cell line (A549) which was subcutaneously implanted into nude mice, resulting in tumor growth at site of implantation. Since these cells do not contain a mutation in the Ha-ras gene, but do express normal Ha-ras, only the AUG-directed oligonucleotide ISIS 2503 was evaluated for anti-tumor activity.

In the first study, phosphorothioate oligonucleotides in saline were administered by intraperitoneal injection at a dosage of 20 mg/kg. Drug treatment was initiated at the time tumors first became visible (28 days following tumor cell inoculation) and treatments were performed every other day. As shown in Figure 16, no effect on tumor growth was observed after treatment with the unrelated control phosphorothioate oligonucleotide ISIS 1082. However, significant inhibition of tumor growth was observed for the Ha-ras-specific oligonucleotide ISIS 2503 (SEQ ID NO: 2). The anti-tumor effects of the Ha-ras compound were first observed 20 days following initiation of drug treatment and continued throughout the duration of the study.

In a second study, phosphorothioate oligonucleotides were prepared in a cationic lipid formulation (DMRIE:DOPE) and administered by subcutaneous injection as described in Example 15. Drug treatment was initiated one week following tumor cell inoculation and was performed three times a week for only four weeks. As was observed in the first study, administration of the Ha-ras-specific compound ISIS 2503 (SEQ ID NO: 2) caused a marked reduction in tumor growth whereas the unrelated control oligonucleotide (ISIS 1082) had no significant effect

(Figure 17). Reduction in tumor volume was first observed 20 days following appearance of visible tumors and continued over time throughout the remainder of the study.

Stability of 2'-modified phosphodiester oligonucleotides in cells: Modification of oligonucleotides to confer nuclease stability is required for antisense activity in cells. Certain modifications at the 2' position of the sugar have been found to confer nuclease resistance sufficient to elicit antisense effects in cells without any backbone modification. As shown in Figure 18, a uniformly 2'-propoxy modified phosphodiester oligonucleotide (SEQ ID NO: 3) was found to inhibit Ha-ras expression in T24 cells, 24 hours after administration, at a level equivalent to a phosphorothioate 2'-deoxyoligonucleotide having the same sequence. Uniform 2'-methoxy phosphodiester oligonucleotide also showed some activity. 2'-pentoxy modifications were found to be at least as active as the 2'-propoxy.

Antisense oligonucleotides active against Ki-ras: Oligonucleotides were designed to be complementary to the 5'-untranslated region, 3'-untranslated region and coding region of the human Ki-ras oncogene. McGrath, J.P. et al., (1983) *Nature* 304, 501-506. Of the latter, oligonucleotides were targeted to codons 12 and 61 which are known sites of mutation that lead to Ki-ras-mediated transformation, and also to codon 38, which is not known to be involved in transformation. The oligonucleotides are shown in Table 8.

Table 8

Antisense Oligonucleotides Complementary to Human Ki-ras

ISIS #	SEQUENCE	TARGET	SEQ ID NO:
6958	CTG CCT CCG CCG CCG CGG CC	5' UTR/5' cap	20
6957	CAG TGC CTG CGC CGC GCT CG	5'-UTR	21
6956	AGG CCT CTC TCC CGC ACC TG	5'-UTR	22
6953	TTC AGT CAT TTT CAG CAG GC	AUG	23
6952	TTA TAT TCA GTC ATT TTC AG	AUG	24
6951	CAA GTT TAT ATT CAG TCA TT	AUG	25
6950	GCC TAC GCC ACC AGC TCC AAC	Codon 12 (WT)	26
6949	CTA CGC CAC CAG CTC CA	Codon 12 (WT)	27
6948	G TAC TCC TCT TGA CCT GCT GT	Codon 61 (WT)	28
6947	CCT GTA GGA ATC CTC TAT TGT	Codon 38	29

6946	GGT AAT GCT AAA ACA AAT GC	3'-UTR	30
6945	GGA ATA CTG GCA CTT CGA GG	3'-UTR	31
7453	TAC GCC AAC AGC TCC	Codon 12 (G→T mut.)	32
7679	TTT TCA GCA GGC CTC TCT CC	5'-UTR/AUG	33

Twelve Ki-ras-specific oligonucleotides were screened for antisense activity against three colon carcinoma cell lines that contain a mutation at codon 12 in the Ki-ras oncogene and evaluated by measurement of Ki-ras mRNA levels. As shown in Figure 19, half of the tested compounds displayed significant activity (at least 40% inhibition) against the Ki-ras transcript, with the most active compounds being targeted to the 5'- and 3'-untranslated regions. However, significant inhibition of Ki-ras expression was also observed for compounds directed against wild type codons 12 and 61. Compounds that displayed significant activity were effective against all three carcinoma cell lines tested.

Dose response analysis of these compounds demonstrated that ISIS 6958 and ISIS 6957, both of which target the 5'-UTR, are the most potent inhibitors of Ki-ras in this series of oligonucleotides. These oligonucleotides were examined for their ability to inhibit proliferation of Ki-ras transformed cell lines. The colon carcinoma cell line SW480 was treated with a single dose of oligonucleotide (200 nM) and cell number was determined over a five-day period. As shown in Figure 20, both Ki-ras specific oligonucleotides were effective inhibitors of proliferation of SW480 cells, with ISIS 6957 (SEQ ID NO: 21) showing greater activity than ISIS 6958 (SEQ ID NO: 20). This difference in activity correlates well with the inhibition of Ki-ras mRNA expression (Figure 19).

Selectivity of inhibition of mutant Ki-ras relative to normal Ki-ras: Oligonucleotides targeted to Ki-ras have been examined for their ability to selectively inhibit mutant Ki-ras relative to normal Ki-ras. Two cell lines were employed: the SW480 cell line that expresses mutant Ki-ras (codon 12, G to T transversion) and a cell line (HeLa) that expresses normal Ki-ras. Two oligonucleotides were tested: ISIS 6957, SEQ ID

NO: 21, a 20mer phosphorothioate targeted to the 5'-untranslated region of Ki-ras, and ISIS 7453, SEQ ID NO: 32, a 15mer phosphorothioate targeted to the Ki-ras codon 12 region. Ki-ras mRNA levels were measured 24 hours after treatment. The codon 12-directed compound was effective in the cell line expressing mutant Ki-ras. However, as shown in Figure 21, the Ki-ras oligonucleotide targeted to the 5'-untranslated region was a potent inhibitor of Ki-ras expression in both cell lines. Selectivity for mutant Ki-ras was found to be dependent on oligonucleotide concentration and affinity for the RNA target.

Ki-ras oligonucleotides with deoxy gaps: Phosphorothioate oligonucleotides (SEQ ID NO: 21, targeted to the 5'-untranslated region of Ki-ras) were synthesized with 2'-O-methyl modifications flanking central 2'-deoxy gap regions of 6 or 8 nucleotides in length. Both gapped oligonucleotides were active against Ki-ras expression as determined by Northern blot analysis. A uniformly 2'-O-methylated compound (no deoxy gap) was inactive. An additional oligonucleotide, ISIS 7679 (SEQ ID NO: 33, complementary to the 5' untranslated/AUG region of Ki-ras), was also found to be active when synthesized with a 6- or 8- nucleotide deoxy gap.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed, however the actual synthesis of the oligonucleotides are well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

The oligonucleotides of this invention are designed to be complementary to, and thus hybridizable with, messenger RNA derived from the H-ras gene. Such hybridization, when accomplished, interferes with the normal roles of the messenger RNA to cause a loss of its function in the cell. The functions

of messenger RNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to interfere with expression of the H-ras gene. Some oligonucleotides of this invention are designed to activate RNAse H cleavage of the ras mRNA.

The protein products of the other mammalian ras genes, N-ras and K-ras, are identical to H-ras over the first 85 amino acids. The nucleic acid sequences of the three ras genes, while not identical, are known, and persons of ordinary skill in the art will be able to use this invention as a guide in preparing oligonucleotides specifically hybridizable with the N-ras and K-ras genes. While the preferred embodiments of this invention relate to antisense oligonucleotides specifically hybridizable with codon 12 of the H-ras mRNA, this invention can be used by persons skilled in the art as a guide in preparing oligonucleotides specifically hybridizable with other point mutations of the ras gene, particularly the well defined point mutations at codon 12, codon 13 and codon 61 of H-ras, N-ras and K-ras, the sequences of which are known.

Figure 22 illustrates the chemical structural aspects of certain preferred chimeric oligonucleotides of the invention. The left hand structure illustrates an oligonucleotide having certain heteroatom linkage abbreviated as MMI linkages connecting each of the sugar-nucleosidic base units together. Specific methods for preparing these MMI linkages are taught in United States patents 5,378,825, that issued on January 3, 1995; 5,386,023, that issued on January 31, 1995; 5,489,243, that issued on February 6, 1996; 5,541,,307, that issued on July 30, 1996; 5,618,704, issued April 8, 1997; and 5,623,070, issued April 22, 1997; each of which is incorporate herein by reference. MMI is an abbreviation for methylene(methylimino) that in turn is a shorten version of the more complex chemical nomenclature "3'-de(oxyphosphinico)-

3'[methylene(methylimino)]." Irrespective of chemical nomenclature, the linkages are as described in these patents. The linkages of these patents have also been described in various scientific publications by the inventors and their co-authors including Bhat *et al.*, *J. Org. Chem.*, **1996**, *61*, 8186-8199, and the various references cited therein.

Illustrated in the center of Figure 22 are certain oligonucleotides that have staggered or alternating linkages. These are formed from alternating MMI and phosphodiester or phosphorothioate linkages. Illustrated on the right hand side of Figure 22 are oligonucleotides that have chimeric structures formed by a region of phosphorothioate linkages in a central "gap" flanked by the mixed or alternating, i.e. staggered, linkages of the center of the figure.

In the MMI containing oligonucleotides of Figure 22, except for those in the "gap" region on the left hand side of the figure, the remainder of the nucleoside units of the "flank" regions of the various oligonucleotides are illustrated as a 2'-O-methyl nucleoside units, i.e. 2'-OMe nucleosides. Other 2'-substituted nucleoside units for inclusion in the oligonucleotides of the invention include 2'-fluoro, 2'-O-alkyl and 2'-O-substituted alkyl nucleosides. A preferred substituent is an alkoxy subsituents, i.e. an ethers. Most preferred is are 2'-O-methoxyethyl substituted nucleosides, see Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

Various oligonucleotides of SEQ ID NO. 2 having specific chemical structures as illustrated in Figure 22 were tested for their ability to inhibit production of H-ras mRNA in T-24 cells. For these test, T-24 cells were plated in 6-well plates and then treated with various escalating concentrations of oligonucleotide in the presence of cationic lipid (Lipofectin, GIBCO) at the ration of 2.5 μg/ml Lipofectin per 100 nM oligonucleotide. Oligonucleotide treatment was carried out in serum free media for 4 hours. Eighteen hours after treatment the total RNA was harvested and analyzed by northern blot for H-ras mRNA and control gene G3PDH. The structures of the oligonucleotides and the test data are shown in Figures 23 and

24. The data is presented in the bar graphs as percent control normalized for the G3PDH signal. As is seen in the Figures 23 and 24, oligonucleotides of the inventions each exhibited a dose responses across the range of doses. The chimeric oligonucleotides that incorporated the MMI nucleoside units had responses equivalent to or better than the phosphorothioate oligonucleotide used as the positive control for these tests with oligonucleotides having 1 or 2 MMI linkages (oligos 14896, 14897 and 14898) in each of the flank regions showing the greatest reduction of H-ras mRNA. The oligo having 3 MMI linkages alternating with phosphorothioate linkages (oligo 14900) was essential equal potent with that of the positive control while the oligo having 3 MMI linkages alternating with phosphodiester linkages (oligo 14899) showed lower potency than that of the positive control. Of equal potency with the positive control was the oligonucleotide (oligo 13920) having 2'-O-methoxyethyl substituents in its flanking regions.

The oligonucleotides of this invention can be used in diagnostics, therapeutics and as research reagents and kits. Since the oligonucleotides of this invention hybridize to the ras gene, sandwich and other assays can easily be constructed to exploit this fact. Furthermore, since the oligonucleotides of this invention hybridize preferentially to the mutant (activated) form of the ras oncogene, such assays can be devised for screening of cells and tissues for ras conversion from wild-type to activated form. Such assays can be utilized for differential diagnosis of morphologically similar tumors, and for detection of increased risk of cancer stemming from ras gene activation. Provision of means for detecting hybridization of oligonucleotide with the ras gene can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of ras or activated ras may also be prepared.

The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

Example 1 Oligonucleotide Synthesis

Substituted and unsubstituted deoxyoligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidate chemistry with oxidation by iodine. For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitation twice out of 0.5 M NaCl solution with 2.5 volumes ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 454 mM Tris-borate buffer, pH=7.0. Oligonucleotides were judged from polyacrylamide gel electrophoresis to be greater than 80% full-length material.

Oligoribonucleotides were synthesized using the automated synthesizer and 5'-dimethoxy-trityl 2'-*tert*-butyldimethylsilyl 3'-O-phosphoramidites (American Bionetics, Hayward, CA). The protecting group on the exocyclic amines of A,C and G was phenoxyacetyl [Wu, T., Oglivie, K.K., and Pon, R.T., *Nucl. Acids Res.* 1989, 17, 3501-3517]. The standard synthesis cycle was modified by increasing the wait step after the pulse delivery of tetrazole to 900 seconds. Oligonucleotides were deprotected by overnight incubation at room temperature in methanolic ammonia. After drying *in vacuo*, the 2'-silyl group was removed by overnight incubation at room temperature in 1 M tetrabutylammoniumfluoride (Aldrich; Milwaukee, WI) in tetrahydrofuran. Oligonucleotides were purified using a C-18 Sep-Pak cartridge (Waters; Milford, MA) followed by ethanol precipitation. Analytical denaturing polyacrylamide electrophoresis demonstrated the RNA oligonucleotides were greater than 90% full length material.

Example 2 ras-Luciferase Reporter Gene Assembly

The ras-luciferase reporter genes described in this study were assembled using PCR technology. Oligonucleotide primers were synthesized for use as primers for PCR cloning of the 5'-regions of exon 1 of both the mutant (codon 12) and non-mutant (wild-type) human H-ras genes. The plasmids pT24-C3, containing the c-H-ras1 activated oncogene (codon 12, GGC→GTC), and pbc-N1, containing the c-H-ras proto-oncogene, were obtained from the American Type Culture Collection (Bethesda, MD). The plasmid pT3/T7 luc, containing the 1.9 kb firefly luciferase gene, was obtained from Clontech Laboratories (Palo Alto, CA). The oligonucleotide PCR primers were used in standard PCR reactions using mutant and non-mutant H-ras genes as templates. These primers produce a DNA product of 145 base pairs corresponding to sequences -53 to +65 (relative to the translational initiation site) of normal and mutant H-ras, flanked by NheI and HindIII restriction endonuclease sites. The PCR product was gel purified, precipitated, washed and resuspended in water using standard procedures.

PCR primers for the cloning of the *P. pyralis* (firefly) luciferase gene were designed such that the PCR product would code for the full-length luciferase protein with the exception of the amino-terminal methionine residue, which would be replaced with two amino acids, an amino-terminal lysine residue followed by a leucine residue. The oligonucleotide PCR primers used for the cloning of the luciferase gene were used in standard PCR reactions using a commercially available plasmid (pT3/T7-Luc) (Clontech), containing the luciferase reporter gene, as a template. These primers yield a product of approximately 1.9 kb corresponding to the luciferase gene, flanked by unique HindIII and BssHII restriction endonuclease sites. This fragment was gel purified, precipitated, washed and resuspended in water using standard procedures.

To complete the assembly of the ras-luciferase fusion reporter gene, the ras and luciferase PCR products were digested with the appropriate restriction endonucleases and cloned by three-part ligation into an expression vector

containing the steroid-inducible mouse mammary tumor virus promotor MMTV using the restriction endonucleases NheI, HindIII and BssHII. The resulting clone results in the insertion of H-ras 5′ sequences (-53 to +65) fused in frame with the firefly luciferase gene. The resulting expression vector encodes a ras-luciferase fusion product which is expressed under control of the steroid-inducible MMTV promoter. These plasmid constructions contain sequences encoding amino acids 1-22 of activated (RA2) or normal (RA4) H-ras proteins fused in frame with sequences coding for firefly luciferase. Translation initiation of the ras-luciferase fusion mRNA is dependent upon the natural H-ras AUG codon. Both mutant and normal H-ras luciferase fusion constructions were confirmed by DNA sequence analysis using standard procedures.

Example 3 Transfection of Cells with Plasmid DNA

Transfections were performed as described by Greenberg, M.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY, with the following modifications. HeLa cells were plated on 60 mm dishes at 5 x 10^5 cells/dish. A total of 10 μg or 12 μg of DNA was added to each dish, of which 1 μg was a vector expressing the rat glucocorticoid receptor under control of the constitutive Rous sarcoma virus (RSV) promoter and the remainder was ras-luciferase reporter plasmid. Calcium phosphate-DNA coprecipitates were removed after 16-20 hours by washing with Tris-buffered saline [50 Mm Tris-Cl (pH 7.5), 150 mM NaCl] containing 3 mM EGTA. Fresh medium supplemented with 10% fetal bovine serum was then added to the cells. At this time, cells were pre-treated with antisense oligonucleotides prior to activation of reporter gene expression by dexamethasone.

Example 4 Oligonucleotide Treatment of Cells

Following plasmid transfection, cells were washed with phosphate buffered saline prewarmed to 37°C and Opti-MEM

containing 5 μg/mL N-[1-(2,3-dioleyloxy)propyl]-N,N,N,-trimethylammonium chloride (DOTMA) was added to each plate (1.0 ml per well). Oligonucleotides were added from 50 μM stocks to each plate and incubated for 4 hours at 37°C. Medium was removed and replaced with DMEM containing 10% fetal bovine serum and the appropriate oligonucleotide at the indicated concentrations and cells were incubated for an additional 2 hours at 37°C before reporter gene expression was activated by treatment of cells with dexamethasone to a final concentration of 0.2 μM. Cells were harvested and assayed for luciferase activity fifteen hours following dexamethasone stimulation.

Example 5 **Luciferase Assays**

Luciferase was extracted from cells by lysis with the detergent Triton X-100 as described by Greenberg, M.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY. A Dynatech ML1000 luminometer was used to measure peak luminescence upon addition of luciferin (Sigma) to 625 μM. For each extract, luciferase assays were performed multiple times, using differing amounts of extract to ensure that the data were gathered in the linear range of the assay.

Example 6 **Melting Curves**

Absorbance vs temperature curves were measured at 260 nm using a Gilford 260 spectrophotometer interfaced to an IBM PC computer and a Gilford Response II spectrophotometer. The buffer contained 100 mM Na^+, 10 mM phosphate and 0.1 mM EDTA, pH 7. Oligonucleotide concentration was 4 μM each strand determined from the absorbance at 85°C and extinction coefficients calculated according to Puglisi and Tinoco, *Methods in Enzymol.* **1989**, 180, 304-325. T_m values, free energies of duplex formation and association constants were obtained from fits of data to a two state model with linear sloping baselines. Petersheim, M. and Turner, D.H., *Biochemistry* **1983**, 22, 256-263. Reported parameters are

averages of at least three experiments. For some oligonucleotides, free energies of duplex formation were also obtained from plots of T_m^{-1} vs $\log_{10}$ (concentration). Borer, P.N., Dengler, B., Tinoco, I., Jr., and Uhlenbeck, O.C., *J. Mol. Biol.*, **1974**, 86, 843-853.

Example 7 Gel Shift Assay

The structured ras target transcript, a 47-nucleotide hairpin containing the mutated codon 12, was prepared and mapped as described in Lima et al., *Biochemistry* **1991**, 31, 12055-12061. Hybridization reactions were prepared in 20 μl containing 100 mM sodium, 10 mM phosphate, 0.1 mM EDTA, 100 CPM of *T7*-generated RNA (approximately 10 pM), and antisense oligonucleotide ranging in concentration from 1 pM to 10 μM. Reactions were incubated 24 hours at 37°C. Following hybridization, loading buffer was added to the reactions and reaction products were resolved on 20% native polyacrylamide gels, prepared using 45 mM tris-borate and 1 mM $MgCl_2$ (TBM). Electrophoresis was carried out at 10°C and gels were quantitated using a Molecular Dynamics Phosphorimager.

Example 8 RNase H Analysis

RNase H assays were performed using a chemically synthesized 25-base oligoribonucleotide corresponding to bases +23 to +47 of activated (codon 12, G→U) H-ras mRNA. The 5′ end-labeled RNA was used at a concentration of 20 nM and incubated with a 10-fold molar excess of antisense oligonucleotide in a reaction containing 20 mM tris-Cl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg tRNA and 4 U RNasin in a final volume of 10 μl. The reaction components were preannealed at 37°C for 15 minutes then allowed to cool slowly to room temperature. HeLa cell nuclear extracts were used as a source of mammalian RNase H. Reactions were initiated by addition of 2 μg of nuclear extract (5 μl) and reactions were allowed to proceed for 10 minutes at 37°C. Reactions were stopped by phenol/chloroform extraction and RNA components were precipitated with ethanol. Equal CPMs were

loaded on a 20% polyacrylamide gel containing 7M urea and RNA cleavage products were resolved and visualized by electrophoresis followed by autoradiography. Quantitation of cleavage products was performed using a Molecular Dynamics Densitometer.

Example 9 ras Transactivation Reporter Gene System

The expression plasmid pSV2-oli, containing an activated (codon 12, GGC→GTC) H-ras cDNA insert under control of the constitutive SV40 promoter, was a gift from Dr. Bruno Tocque (Rhone-Poulenc Sante, Vitry, France). This plasmid was used as a template to construct, by PCR, a H-ras expression plasmid under regulation of the steroid-inducible mouse mammary tumor virus (MMTV) promoter. To obtain H-ras coding sequences, the 570 bp coding region of the H-ras gene was amplified by PCR. The PCR primers were designed with unique restriction endonuclease sites in their 5'-regions to facilitate cloning. The PCR product containing the coding region of the H-ras codon 12 mutant oncogene was gel purified, digested, and gel purified once again prior to cloning. This construction was completed by cloning the insert into the expression plasmid pMAMneo (Clontech Laboratories, CA).

The ras-responsive reporter gene pRDO53 was used to detect ras expression. Owen et al., *Proc. Natl. Acad. Sci. U.S.A.* 1990, 87, 3866-3870.

Example 10 Northern blot analysis of ras expression *in vivo*

The human urinary bladder cancer cell line T24 was obtained from the American Type Culture Collection (Rockville MD). Cells were grown in McCoy's 5A medium with L-glutamine (Gibco BRL, Gaithersburg MD), supplemented with 10% heat-inactivated fetal calf serum and 50 U/ml each of penicillin and streptomycin. Cells were seeded on 100 mm plates. When they reached 70% confluency, they were treated with oligonucleotide. Plates were washed with 10 ml prewarmed PBS and 5 ml of Opti-MEM reduced-serum medium containing 2.5 μl DOTMA.

Oligonucleotide was then added to the desired concentration. After 4 hours of treatment, the medium was replaced with McCoy's medium. Cells were harvested 48 hours after oligonucleotide treatment and RNA was isolated using a standard CsCl purification method. Kingston, R.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY.

The human epithelioid carcinoma cell line HeLa 229 was obtained from the American Type Culture Collection (Bethesda, MD). HeLa cells were maintained as monolayers on 6-well plates in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin. Treatment with oligonucleotide and isolation of RNA were essentially as described above for T24 cells.

Northern hybridization: 10 μg of each RNA was electrophoresed on a 1.2% agarose/formaldehyde gel and transferred overnight to GeneBind 45 nylon membrane (Pharmacia LKB, Piscataway, NJ) using standard methods. Kingston, R.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY. RNA was UV-crosslinked to the membrane. Double-stranded ^{32}P-labeled probes were synthesized using the Prime a Gene labeling kit (Promega, Madison WI). The ras probe was a SalI-NheI fragment of a cDNA clone of the activated (mutant) H-ras mRNA having a GGC-to-GTC mutation at codon-12. The control probe was G3PDH. Blots were prehybridized for 15 minutes at 68°C with the QuickHyb hybridization solution (Stratagene, La Jolla, CA). The heat-denatured radioactive probe (2.5 x 10^6 counts/2 ml hybridization solution) mixed with 100 μl of 10 mg/ml salmon sperm DNA was added and the membrane was hybridized for 1 hour at 68°C. The blots were washed twice for 15 minutes at room temperature in 2x SSC/0.1% SDS and once for 30 minutes at 60°C with 0.1XSSC/0.1%SDS. Blots were autoradiographed and the intensity of signal was quantitated using an ImageQuant PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Northern

blots were first hybridized with the ras probe, then stripped by boiling for 15 minutes in 0.1x SSC/0.1%SDS and rehybridized with the control G3PDH probe to check for correct sample loading.

Example 11 **Antisense oligonucleotide inhibition of proliferation of cancer cells**

Cells were cultured and treated with oligonucleotide essentially as described in Example 10. Cells were seeded on 60 mm plates and were treated with oligonucleotide in the presence of DOTMA when they reached 70% confluency. Time course experiment: On day 1, cells were treated with a single dose of oligonucleotide at a final concentration of 100 nM. The growth medium was changed once on day 3 and cells were counted every day for 5 days, using a counting chamber. Dose-response experiment: Various concentrations of oligonucleotide (10, 25, 50, 100 or 250 nM) were added to the cells and cells were harvested and counted 3 days later. Oligonucleotides 2570, 3985 and 4690 were tested for effects on T24 cancer cell proliferation.

Example 12 **Synthesis of 2-(amino)adenine-substituted oligonucleotides**

Oligonucleotides are synthesized as in Example 1, with the following exception: at positions at which a 2-(amino)adenine is desired, the standard phosphoramidite is replaced with a commercially available 2-aminodeoxyadenosine phosphoramidite (Chemgenes).

Example 13 **Culture of A549 cells**

A549 cells (obtained from the American Type Culture Collection, Bethesda MD) were grown to confluence in 6-well plates (Falcon Labware, Lincoln Park, NJ) in Dulbecco's modified Eagle's medium (DME) containing 1 g glucose/liter and 10% fetal calf serum (FCS, Irvine Scientific, Santa Ana, CA).

Example 14 Oligonucleotide treatment of human tumor cells in nude mice - intraperitoneal injection

Human lung carcinoma A549 cells were harvested and 5 x 10^6 cells (200 μl)were injected subcutaneously into the inner thigh of nude mice. Palpable tumors develop in approximately one month. Phosphorothioate oligonucleotides ISIS 2503 and 1082 (unrelated control) were administered to mice intraperitoneally at a dosage of 20 mg/kg body weight, every other day for approximately ten weeks. Mice were monitored for tumor growth during this time.

Example 15 Oligonucleotide treatment of human tumor cells in nude mice - subcutaneous injection with cationic lipid

Human lung carcinoma A549 cells were harvested and 5 x 10^6 cells (200 μl) were injected subcutaneously into the inner thigh of nude mice. Palpable tumors develop in approximately one month. Phosphorothioate oligonucleotides ISIS 2503 and the unrelated control oligonucleotide 1082 (dosage 5 mg/kg), prepared in a cationic lipid formulation (DMRIE/DOPE, 60 mg/kg) were administered to mice subcutaneously at the tumor site. Drug treatment began one week following tumor cell inoculation and was given twice a week for only four weeks. Mice were monitored for tumor growth for a total of nine weeks.

Example 16 Stability of 2' modified oligonucleotides in T24 cells

T24 bladder cancer cells were grown as described in Example 10. Cells were treated with a single dose (1 μM) of oligonucleotide and assayed for Ha-ras mRNA expression by Northern blot analysis 24 hours later. Oligonucleotides tested were analogs of ISIS 2570 (SEQ ID NO: 3), a 17mer targeted to Ha-ras codon 12.

Example 17 Activity of Ki-ras oligonucleotides against three colon carcinoma cell lines

Human colon carcinoma cell lines Calu 1, SW480 and SW620 were obtained from the American Type Culture Collection (ATCC) and cultured and maintained as described for HeLa cells in Example 10. Cells were treated with a single dose of oligonucleotide (200 mM) and Ki-ras mRNA expression was measured by Northern blot analysis 24 hours later. For proliferation studies, cells were treated with a single dose of oligonucleotide (200 nM) at day zero and cell number was monitored over a five-day period.

Example 18 Oligonucleotide inhibition of mutant vs. wild-type Ki-ras

SW480 cells were cultured as in the previous example. HeLa cells were cultured as in Example 10. Cells were treated with a single dose (100 nM) of oligonucleotide and mRNA levels were determined by Northern blot analysis 24 hours later.

EXAMPLES 19-35

General information

Unless otherwise noted, materials were obtained from commercial suppliers and were used as provided. 2'-*O*-Methyl-5-methyluridine, 5'-*O*-(4,4'-dimethoxytriphenylmethyl)-2'-*O*-methyl-5-methyluridine, 5'-*O*-(4,4'-dimethoxytriphenylmethyl)-*N*-2-isobutyryl-2'-*O*-methylguanosine, and *N*-6-benzoyl-5'-*O*-(4,4'-dimethoxytriphenylmethyl)-2'-*O*-methyladenosine were purchased from RI Chemicals, CA (714-288-1548). 2'-*O*-Methylguanosine and 2'-*O*-methyladenosine were purchased from Summit Pharmaceuticals Corp., N. J. (201-585-9687).

NMR Spectroscopy. ^{1}H NMR spectra for all compounds were recorded either at 399.94 MHz on a Varian Unity 400 NMR spectrometer or at 199.975 MHz on a Varian Gemini 200 NMR spectrometer. All multi-dimensional and variable temperature spectra were collected on the Unity 400 NMR. The compound **19d** was studied at a concentration of 1.6mM in 600μl DMSO-d_6. One-dimensional ^{1}H NMR spectra were recorded at the sample

temperatures 293, 310, 333 , and 353 K. All one-dimensional measurements were performed under the same conditions and processed identically: 5200 Hz sweep width, 32K time domain, zero-filling to 64K. Where possible, the data were studied unapodized, but in some instances the standard Varian VnmrS resolution enhancement method was invoked, using a line broadening of -1.01 and a gaussian filter of 0.945. All subspectra were observed to be first order. Proton resonances were assigned from two-dimensional spectra run at 310 and 353 K. TOCSY and NOESY spectra were run at 310 K with a 20 ms spin-lock and a 650 ms mixing time, respectively, with 1k by 512 complex points being collected in a phase-sensitive manner. Similar experiments were carried out at 353 K, with 2k by 512 complex points collected in a phase-sensitive manner. In each case, the data was apodized with a squared cosine and zero-filled to 2k x 1k complex points before fourier transformation. The ^{13}C spectra were recorded on the same instrument, at a frequency of 100.57 MHz, using a sweep width of 30441 Hz, an acquisition time of 0.50 s, and processed by apodizing with 2 Hz line-broadening and zero-filling to 32K. The ^{13}C spectrum was assigned with the aid of a $^{1}H\{^{13}C\}$ HMQC at 293 K. For this experiment 1K complex points were collected in the directly-detected dimension over a sweep width of 3200 Hz. A total of 240 increments were collected in a phase-sensitive manner in the indirectly-detected dimension, over a sweep-width of 22 kHz. The data was apodized with a squared cosine in the ^{1}H dimension and a gaussian in t_1.

One-dimensional ^{1}H spectra for **12h**, **12i**, **12j**, **18a**, **20a**, and **20b** were all recorded at 293 K using identical parameters for acquisition and processing: 14336 complex points were collected over a sweep width of 7 kHz. Data was zero-filled to 64K points and apodized with 0.30 Hz line-broadening before fourier transform. Two-dimensional spectra were acquired with sweep widths adjusted to optimize resolution across the spectral range of each sample, ranging from 4 kHz to 5200 Hz in each dimension. Data sets of 1K by 256 points were acquired for **12h** and **12j**, 1K by 512 points for **12i** and **20b**. Data sets

of 2K by 512 data points were collected for **18** and **20b** to allow adequate resolution. Data was collected at 313 K for **20a** and **20b** to enhance chemical shift dispersion. All data was phase-sensitive, processed under conditions otherwise identical to the methods used in the TOCSY and NOESY work outlined above. ^{1}H NMR data for **19b** was collected at 310 K in deuterium oxide, using the same series of experiments described above. Other general experimental procedures were carried out as described previoulsy in Perbost, M.; Hoshiko, T.; Morvan, F.; Swayze, E.; Griffey, R. H.; Sanghvi, Y. S., *J. Org. Chem.* **1995**, *60*, 5150-5156.

EXAMPLE 19

General Procedure A, Synthesis of 3'-iodo-2'-*O*-methyl Nucleosides (11→12)

To a solution of pyridine (4.3 eq) in dry CH_2Cl_2 (5 mL/mmol) at -10 °C under an inert atmosphere is added a solution of trifluoromethanesulfonic anhydride (2 eq) in CH_2Cl_2 (5 mL/mmol) dropwise over 0.5 h. After stirring an additional 0.25 h, a solution of appropriately protected 3'-hydroxyl nucleoside (1 eq, azeotroped with dry pyridine) in dry CH_2Cl_2 (5 mL/mmol) was added dropwise over 0.5 h, and the solution stirred at -10 °C for an additional 2 h. The reaction mixture was diluted with an equal volume of ice cold 10% aqueous $NaHCO_3$ with vigorous stirring, and the mixture was allowed to warm to rt. The organic phase was removed, dried ($MgSO_4$), diluted with toluene (5 mL/mmol), concentrated, and azeotroped with dry toluene (3 x 5 mL/mmol). To the resulting foam was added Bu_4NI (2 eq) and dry toluene (30 mL/mmol), and the resulting mixture heated for 0.5 h at 80 °C in an oil bath with vigorous stirring, at which point all solid had dissolved, and a dark red oil separated. The resulting mixture was dissolved by dilution with EtOAc, then washed with water (15 mL/mmol), 1:1 5% aqueous $NaHSO_3$ and 5% aqueous Na_2SO_3 (2 x 15 mL/mmol), dried ($MgSO_4$), concentrated, and chromatographed to provide the iodide.

EXAMPLE 20

3'-Deoxy-5'-*O*-(4,4'-dimethoxytriphenylmethyl)-3'-iodo-2'-*O*-methyl-5-methyluridine (12a)

From 5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-methyl-5-methyluridine (5.75 g, 10 mmol) according to general procedure A was obtained 4.41 g (64%) of **12a** after chromatography (30% to 50% EtOAc/hexane): R_f 0.38 (50% EtOAc/hexane); ^1H NMR ($CDCl_3$) δ 9.91 (s, 1H), 7.55-7.20 (m, 10H), 6.85 (d, 4H), 5.85 (s, 1H, H-1'), 4.37 (s, 1H), 4.31 (d, 1H), 3.93 (m, 1H), 3.79 (s, 6H), 3.60-3.50 (m, 1H), 3.56 (s, 3H), 3.20 (m, 1H), 1.83 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 164.16, 158.53, 150.35, 144.23, 136.14, 135.44, 135.25, 130.06, 128.12, 127.77, 126.88, 113.06, 110.06, 92.48, 90.17, 86.55, 80.00, 68.56, 58.32, 55.09, 26.84, 12.32. Anal. Calcd for $C_{32}H_{33}N_2O_7I \cdot 0.4\ CH_2Cl_2$: C, 54.16; H, 4.74; N, 3.90. Found: C, 54.27; H, 4.58; N, 3.73.

EXAMPLE 21

3'-Deoxy-5'-*O*-(4,4'-dimethoxytriphenylmethyl)-3'-iodo-2-*N*-isobutyryl-2'-*O*-methylguanosine (12c)

From 5 g (8.26 mmol) of **11c** according to general procedure A was obtained 3.8g (59%) of **12b** after chromatography (80% EtOAc/hexane): ^1H NMR (DMSO-d_6) δ 12.14 (s, 1H), 11.67 (s, 1H), 7.96 (s, 1H), 7.42-7.20 (m, 9H), 7.17-6.86 (m, 4H), 5.85 (d, J=2.1 Hz, 1H), 4.74 (bs, 2H), 3.87-3.86 (m, 1H), 3.73 (s, 6H), 3.32-3.08 (m, 5H), 2.85-2.70 (m, 1H), 1.12-1.03 (m, 6H); MS (FAB^+) *m/e* 780 (M+H). Anal. Calcd for $C_{36}H_{38}N_5O_7I \cdot 0.25\ C_6H_{14}$: C, 56.25; H, 5.16; N, 8.75. Found: C, 56.48; H, 5.24; N, 8.68.

EXAMPLE 22

6-*N*-Benzoyl-3'-deoxy-5'-*O*-(4,4'-dimethoxytriphenylmethyl)-3'-iodo-2'-*O*-methyladenosine (12k)

From *N*-6-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-methyladenosine (0.69 g, 1 mmol) according to general procedure A was obtained 0.47 g (58%) of **12k** after chromatography (50% to 70% EtOAc/hexane): R_f 0.34 (50% EtOAc/hexane); ^1H NMR ($CDCl_3$) δ 9.12 (s, 1H), 8.81 (s, 1H), 8.37 (s, 1H), 8.03 (d,

2H), 7.65-7.15 (m, 9H), 6.85 (d, 4H), 6.18 (s, 1H, H-1'), 4.68 (s, 1H), 4.39 (d, 1H), 3.94 (dd, 1H), 3.80 (s, 6H), 3.66 (dd, 1H), 3.63 (s, 3H), 3.21 (dd, 1H); ^{13}C NMR ($CDCl_3$) δ 164.52, 158.61, 158.57, 152.71, 151.09, 149.37, 144.36, 141.46, 135.64, 135.35, 133.61, 132.72, 130.10, 130.03, 128.81, 128.10, 127.86, 127.79, 126.96, 123.38, 113.16, 92.76, 89.73, 86.61, 80.48, 68.58, 58.38, 55.20, 26.60. Anal. Calcd for $C_{39}H_{36}N_6O_6I\cdot0.4$ $CH_2Cl_2\cdot0.5$ EtOAc: C, 56.79; H, 4.70; N, 8.00. Found: C, 56.60; H, 4.64; N, 8.01.

EXAMPLE 23

5'-*O*-(tert-Butyldiphenylsilyl)-3'-deoxy-3'-iodo-5-methyl-2'-*O*-methyluridine (12e)

A solution of **11e** (7.0 g, 13.7 mmol) and dry pyridine (1.9 g, 24.6 mmol) in anhydrous CH_2Cl_2 (80 mL) was cooled under vigorous stirring to -10 °C in an inert atmosphere for about 20 min. A solution of triflic anhydride (5.1 g, 17.7 mmol) in CH_2Cl_2 (30 mL) was slowly added via a syringe (15 min.). The reaction was complete in 35 min. after the addition. To this cold solution was added MeOH (1 mL) and then saturated $NaHCO_3$ and the organic layer was separated and washed with brine (2 x 15 mL) followed by drying over Na_2SO_4 . Solvent was removed under reduced pressure and the residue azeotroped with toluene (2 x 20 mL). The residual gum was dissolved in *p*-xylene (100 mL) and to this was added Bu_4NI (5.6 g,15.1 mmol), and the reaction mixture heated at 140 °C in a preheated bath. The reaction was complete in 30 min. It was cooled to rt, then diluted with EtOAc (250 mL), and washed with 5% aqueous solution of sodium sulfite and sodium bisulfite (1:1). The organic layer was dried over Na_2SO_4 and concentrated followed by flash chromatography on silica gel using 60% EtOAc/hexane. Removal of the solvent furnished 6.8 g (80%) of **12e** as a colorless foam: ^1H NMR (DMSO-d_6) δ 11.50 (s, 1H), 7.23-7.43 (m, 11H), 5.78 (d, J=4.0 Hz, 1H), 4.6 (bs,1H), 4.40-4.43 (m, 1H), 3.89 (bs, 2H), 3.42 (s, 3H), 1.59 (s, 3H), 1.04 (s, 9H);

MS (FAB^+) *m/e* 621 (M+H). Anal. Calcd for $C_{27}H_{33}N_2O_5SiI$: C, 52.26; H, 5.36; N, 4.51. Found: C, 52.55; H, 5.41; N, 4.48.

EXAMPLE 24

5′-*O*-(tert-Butyldiphenylsilyl)-3′-deoxy-6-*N*-(dimethylamino)methylene-3′-iodo-2′-*O*-methyladenosine (12f)

From **11h** (5.3g, 9.2 mmol), pyridine (3.65 g, 46.1 mmol), triflic anhydride (3.0 g, 11 mmol) and Bu_4NI (5.2 g, 14.0 mmol) according to the method used for **12e** was obtained 3.8 g (60%) of **12f** after chromatography on silica gel using 80:15:3-5% MeOH in EtOAc:hexane: ^1H NMR (DMSO-d_6) δ 8.93 (s, 1H), 8.45 (s, 1H), 8.24 (s, 1H), 7.73-7.39 (m, 10H), 6.07 (d, *J*=2.8 Hz, 1H), 4.89-4.87 (m, 1H), 4.76-4.74 (m, 1H), 3.98-3.93 (m, 3H), 3.46 (s, 3H), 3.21 (s, 3H), 3.14 (s, 3H), 1.03 (s, 9H); MS (FAB^+) *m/e* 685 (M+H). Anal. Calcd for $C_{30}H_{37}N_6O_3SiI$. 0.25 C_6H_{12} : C, 53.61; H, 5.71; N, 11.91. Found: C, 53.20; H, 5.58; N, 12.24.

EXAMPLE 25

5′-*O*-(tert-Butyldiphenylsilyl)-3′-deoxy-2-*N*-(dimethylamino)methylene-3′-iodo-2′-*O*-methylguanosine (12g)

From **11j** (5.0 g, 8.46 mmol), pyridine (3.36 g, 46 mmol), triflic anhydride (3 g, 11 mmol) and Bu_4NI (5.2 g, 14.0 mmol) according to the method used for **12e** was obtained 2.5 g (43%) of **12g** after chromatography on silica gel using 80:15:3-5% MeOH in EtOAc:hexane: ^1HNMR (DMSO-d_6) δ 11.43 (s, 1H), 8.58 (s, 1H), 7.87 (s, 1H), 7.70-7.64 (m, 10H), 5.90 (d, *J*=2 Hz, 1H), 4.88 (bs, 1H), 4.70 (bs, 1H), 3.46 (s, 3H), 3.12 (s, 3H), 3.04 (s, 3H), 1.02 (s, 9H; MS (FAB^+) *m/e* 701 (M+H). Anal. Calcd for $C_{30}H_{37}N_6O_4SiI$: C, 51.43; H, 5.32; N, 11.99. Found: C, 51.56; H, 5.37; N, 11.98.

EXAMPLE 26

General Procedure B, Mitsunobu Reaction of Nucleosides (10-13)

A mixture of the appropriate nucleoside (1 eq.), triphenylphosphine (1.15 eq.) and *N*-hydroxyphthalimide (1.15 eq.) was dried under vacuum over P_2O_5 for 18 h prior to use. To this mixture under an inert atmosphere is added dry THF (for T) or DMF (for A and G^{dmf}) (10 mL/mmol), then diethylazodicarboxylate (1.15 eq.) dropwise with stirring to the solution at rt. The rate of addition is maintained such that the resulting deep red coloration is just discharged before addition of the next drop. After the addition is complete (5-45 min., depending on starting nucleoside and scale), the reaction is stirred until shown to be complete by TLC. The solvent was evaporated *in vacuo* to approximately 1/3 the starting volume, and if a solid was obtained (T, G^{dmf}) it was collected, otherwise (A) the resulting solution was partitioned between water and ether, the resulting solid collected, washed well with ether, and dried to yield the desired product. This material was pure in some cases, but usually contained traces of triphenylphosphine oxide and/or diethyl hydrazinedicarboxylate, which could be removed by column chromatography. However, these impurities did not interfere with the subsequent reactions (general procedure C), and the crude material could therefore be carried directly to the next step.

EXAMPLE 27

2′-*O*-Methyl-5′-*O* -phthalimido-5-methyluridine (13a)

2′-*O*-Methyl-5-methyluridine (4.08 g, 15 mmol) was reacted according to general procedure B. After the reaction was complete (by TLC), the solids were collected, washed well with ether, and dried to yield 3.06 g (49%) of pure product. The combined filtrates were concentrated, then purified by column

chromatography (0 to 4% $MeOH/CH_2Cl_2$) to provide an additional 2.50 g (40%), giving a combined yield of 5.56 (89%) of **13a**: R_f 0.25 (5% $MeOH/CH_2Cl_2$); ^1H NMR (DMSO-d_6) δ 11.36 (s, 1H), 7.87 (br s, 4H), 7.58 (s, 1H), 5.89 (d, J = 5.7 Hz, 1H), 5.43 (d, J = 5.7 Hz, 1H), 4.5-3.8 (m, 6H), 3.34 (s, 3H), 1.79 (s, 3H). Anal. Calcd for $C_{19}H_{19}N_3O_8 \cdot 0.5\ H_2O$: C, 53.52; H, 4.73; N, 9.85. Found: C, 53.51; H, 4.49; N, 9.84.

EXAMPLE 28

2′-*O*-Methyl-5′-*O* -phthalimidoadenosine (13b)

From 2′-*O*-methyl-adenosine (50.0 g, 178 mmol) according to general procedure B was obtained 74.3 g (98%) of product after partitioning the concentrated reaction mixture between ice water (3 L) and ether (3 L), and collection and drying of the resulting solid. The product contains traces (0.125 eq by ^1H NMR and combustion analysis) of $Ph_3P{=}O$, which doesn't interfere in the silylation reaction: R_f 0.38 (10% $MeOH/CH_2Cl_2$); ^1H NMR (DMSO-d_6) δ 8.36 (s, 1H), 8.12 (s, 1H), 7.81 (s, 4H), 6.02 (d, J=5.0 Hz, 1H), 5.50 (d, J=5.0 Hz, 1H), 4.6-4.3 (m, 4H), 4.3-4.2 (m, 1H), 3.33 (s, 3H); HRMS (FAB^+, CsI/NBA) calcd for $C_{19}H_{18}N_6O_6{+}Cs^+$ 559.0342, found 559.0355. Anal. Calcd for $C_{19}H_{18}N_6O_6 \cdot 0.125\ Ph_3P{=}O$ (evident in ^1H NMR): C, 55.34; H, 4.34; N, 18.22. Found: C, 55.07; H, 4.45; N, 17.98.

EXAMPLE 29

2-*N*-(Dimethylamino)methylene-2′-*O*-methyl-5′-phthalimidoguanosine (13c)

2-*N*-(Dimethylamino)methylene-2′-*O*-methylguanosine (**10f**, 6.39 g, 18.2 mmol) was exhaustively dried (0.01 mm Hg, 45 °C, several days over P_2O_5), then reacted according to general procedure B. After the reaction was complete (by TLC), it was reduced to 1/3 its original volume, and the solid was collected and dried to yield 8.85 g (93%) of hydrated **13c**: R_f 0.23 (10% $MeOH/CH_2Cl_2$);. ^1H NMR (DMSO-d_6) δ 11.39 (s, 1H), 8.53 (s, 1H),

8.05 (s, 1H), 7.84 (br s, 4H), 5.92 (d, J = 5.8 Hz, 1H), 5.52 (d, J = 5.3 Hz, 1H), 4.5-4.1 (m, 5H), 3.14 (s, 3 H), 3.01 (s, 3H), 2.88 (s, 3 H); HRMS (FAB^+, CsI/NBA) calcd for $C_{22}H_{23}N_7O_7+Cs^+$ 630.0713, found 630.0725. Anal. Calcd for $C_{22}H_{23}N_7O_7 \cdot 1.5\ H_2O$: C, 50.38; H, 5.00; N, 18.69. Found: C, 50.57; H, 5.12; N, 18.81.

EXAMPLE 30

General Procedure C, Silylation of Nucleosides (13-14)

The requisite nucleoside (1 eq, dried over P_2O_5 *in vacuo*) and imidazole (4.5 eq) were dissolved in dry DMF (5 mL/mmol) under an inert atmosphere, and TBDPSCl (1.5 eq) was added. The reaction was stirred at room temperature until complete by TLC (16-48 h), then poured into EtOAc (15 mL/mmol) and water (15 mL/mmol). The organic layer was washed with water (3 x 10 mL/mmol), dried ($MgSO_4$), and concentrated. On large scale, it proved more convenient to concentrate the crude reaction mixture to a thin syrup and partition the product between ether and water, then collect the resulting solid. This material could be purified by column chromatography, however the only impurities present were silyl by-products, which did not interfere in the subsequent conversion to the oximes *via* general procedure D.

EXAMPLE 31

3′-*tert*-Butyldiphenylsilyl-2-*N*-(dimethylamino)methylene-2′-*O*-methylguanosine (14c)

From **13c** (8.85 g, 16.9 mmol) according to general procedure C was obtained 10.6 g (84%) of **14c** after filtration (0 to 10% MeOH/CH_2Cl_2) of the crude material through a plug of silica: R_f 0.36 (10% MeOH/CH_2Cl_2);. ^1H NMR (DMSO-d_6) δ 11.35 (s, 1H), 8.43 (s, 1H), 8.04 (s, 1H), 7.84 (br s, 4H), 7.5-7.7 (m, 4H), 7.3-7.4 (m, 6H), 6.02 (d, J = 6.1 Hz, 1H), 4.5-4.7 (m, 1H), 4.0-4.5 (m, 4H) 3.05 (s, 3 H), 3.03 (s, 3H), 2.88 (s, 3

H), 1.06 (s, 9H); HRMS (FAB^+, CsI/NBA) calcd for $C_{38}H_{41}N_7O_7Si+Cs^+$ 868.1891, found 868.1899. Anal. Calcd for $C_{38}H_{41}N_7O_7Si \cdot 0.5\ H_2O$: C, 61.27; H, 5.68; N, 13.16. Found: C, 61.27; H, 5.82; N, 13.38.

EXAMPLE 32

General Procedure D, Synthesis of Nucleoside 5′-Formaldoximes (14-15)

The crude material obtained from the silylation of the corresponding 5′-*O*-phthalimido nucleoside was dissolved in dry CH_2Cl_2 (10 mL/mmol), and methylhydrazine (1.2 eq) was added dropwise at -10 to 0 °C. After 1 h, the mixture was filtered, the filtrates washed with CH_2Cl_2, and the combined organics washed with water, brine, dried (Na_2SO_4), and the solution concentrated to yield the 5′-*O*-amino nucleoside, which was used immediately without further purification. (The 5′-*O*-amino nucleosides can be purified by column chromatography, however we have observed that they tend to decompose upon standing). This residue was dissolved in 1:1 EtOAc/MeOH (15 mL/mmol), formaldehyde (20 % w/w aqueous, 1.1 eq) was added, and the mixture stirred 1 h at room temperature. The solution was concentrated, then chromatographed or crystallized to provide the 3′-silyl-5′-formaldoxime.

EXAMPLE 33

3′-*O*-*tert*-Butyldiphenylsilyl-2-*N*-(dimethylamino)methylene-2′-*O*-methyl-5′-*O*-(methyleneimino)guanosine (15e)

From **14c** (10.6 g, 14.4 mmol) according to general procedure D was obtained 9.21 g of **15e** in essentially quantitative yield after filtration (0 to 10% $MeOH/CH_2Cl_2$) of the crude material through a plug of silica: R_f 0.43 (10% $MeOH/CH_2Cl_2$);. ^1H NMR (DMSO-d_6) δ 11.19 (s, 1H), 8.47 (s, 1H), 7.94 (s, 1H), 7.5-7.7 (m, 4H), 7.4-7.5 (m, 6H), 6.96 (d, J=7.27 Hz, 1H), 6.55 (d, J=7.27 Hz, 1H), 6.02 (d, J = 6.13 Hz, 1H),

4.4-4.5 (m, 1H), 4.0-4.3 (m, 4H) 3.06 (s, 3 H), 3.01 (br s, 6H), 1.06 (s, 9H) ppm. Anal. Calcd for $C_{31}H_{39}N_7O_5Si \cdot 0.5\ H_2O$: C, 59.40; H, 6.43; N, 15.64. Found: C, 59.59; H, 6.33; N, 15.61.

EXAMPLE 34

General Procedure E, Radical Coupling Reaction

The appropriate 3′-deoxy-3′-iodo-nucleoside (1 eq) and 5′-formaldoxime (3 eq) were azeotroped with dry benzene (10 mL/mmol), and bis(trimethystannyl)benzopinacolate (2 eq) and dry benzene (5 mL/mmol) were added. The mixture was degassed (argon, 0.5 h), and heated to 80 °C in an oil bath for 16-24 h, at which point all iodide had been consumed (by TLC). The solution was allowed to cool, and stirred vigorously with EtOAc (50 mL/mmol) and 10% aqueous KF (20 mL/mmol) for 2 h. The organic layer was washed with 5% aqueous $NaHCO_3$ (20 mL/mmol), dried ($MgSO_4$), concentrated, dissolved in the minimum amount of CH_2Cl_2, and applied to a column of silica. Elution with 30% EtOAc/hexane (5 column volumes) provided tin by-products and benzophenone. The unreacted oxime (75-99% of unreacted material recovered), and the hydroxylamino linked dimer were then obtained by continued elution with the appropriate solvent system.

EXAMPLE 35

3′-De(oxyphosphinico)-3′-(methyleneimino)-5′-*O*-(triphenylmethyl)thymidylyl-(3′→5′)-3′-*O*-(*tert*-butyldiphenylsilyl)-2′-deoxyadenosine (9d)

From 297 mg (0.5 mmol) of **6b** and 775 mg (1.5 mmol) of **7b** according to general procedure F was obtained 515 mg (83% of unreacted material) of the starting oxime, and 291 mg (59%) of **9d** after elution of the column with a gradient of 0% to 5% MeOH in 4:1 EtOAc/hexane: R_f 0.41 (5% MeOH in 4:1 EtOAc/hexane); ^1H NMR ($CDCl_3$) δ 12.50 (s, 1H), 8.34 (s, 1H), 8.23 (s, 1H),

7.70-7.10 (m, 26H), 6.55 (t, *J*=6.8 Hz, 1H), 6.45 (bs, 2H), 6.08 (t, *J*=5.6 Hz, 1H), 5.98 (s, 1H), 4.50 (m, 1H), 4.07 (m, 1H), 3.94 (m, 1H), 3.47 (m, 2H), 3.28 (dd, 1H), 3.20 (dd, 1H), 2.84 (m, 2H), 2.45 (m, 3H), 2.19 (t, 2H), 1.52 (s, 1H), 1.09 (s, 9H). Anal. Calcd for $C_{56}H_{60}N_8O_7Si \cdot 0.5\ H_2O$: C, 67.65; H, 6.18; N, 11.27. Found: C, 67.77; H, 6.06; N, 11.35.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ISIS Pharmaceuticals, Inc. et al.

(ii) TITLE OF INVENTION:

ANTISENSE INHIBITION OF ras GENE WITH CHIMERIC AND ALTERNATING OLIGONUCLEOTIDES

(iii) NUMBER OF SEQUENCES: 33

(iv) CORRESPONDENCE ADDRESS:

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(B) STREET: One Liberty Place, 46th Floor

(C) CITY: Philadelphia

(D) STATE: PA

(E) COUNTRY: USA

(F) ZIP: 19103

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: PC-DOS

(D) SOFTWARE: WORDPERFECT 6.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: n/a

(B) FILING DATE: herewith

(C) CLASSIFICATION:

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(B) FILING DATE: 30 APRIL 1997

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(viii) ATTORNEY/AGENT INFORMATION:

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

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CTTATATTCC GTCATCGCTC 20

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TCCGTCATCG CTCCTCAGGG 20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCACACCGAC GGCGCCC 17

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCCACACCGA CGGCGCCCA 19

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCCCACACCG ACGGCGCCCA C 21

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TGCCCACACC GACGGCGCCC ACC 23

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TATTCCGTCA TCGCTCCTCA 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGACG 5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCGACGG 7

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

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ACCGACGGC 9

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

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(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CACCGACGGC G 11

(2) INFORMATION FOR SEQ ID NO: 12:

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(A) LENGTH: 13

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

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ACACCGACGG CGC 13

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CACACCGACG GCGCC 15

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCACACCGAC GGCGCC 16

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CACACCGACG GCGCCC 16

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCCACACCGA CGGCGCCC 18

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCACACCGAC GGCGCCCA 18

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TTGCCCACAC CGACGGCGCC CACCA 25

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CCACACCGCC GGCGCCC 17

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20

CTGCCTCCGC CGCCGCGGCC 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CAGTGCCTGC GCCGCGCTCG (20)

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AGGCCTCTCT CCCGCACCTG (20)

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

TTCAGTCATT TTCAGCAGGC (20)

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TTATATTCAG TCATTTTCAG (20)

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CAAGTTTATA TTCAGTCATT (20)

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GCCTACGCCA CCAGCTCCAA C (21)

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GTACGCCACC AGCTCCA (17)

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GTACTCCTCT TGACCTGCTG T (21)

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CCTGTAGGAA TCCTCTATTG T (21)

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GGTAATGCTA AAACAAATGC (20)

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GGAATACTGG CACTTCGAGG (20)

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

TACGCCAACA GCTCC (15)

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

TTTTCAGCAG GCCTCTCTCC (20)

What is claimed is:

1. An oligonucleotide having at least one portion comprising at least one CH_2-NH-O-CH_2, CH_2-N(CH_3)-O-CH_2, CH_2-O-N(CH_3)-CH_2, CH_2-N(CH_3)-N(CH_3)-CH_2 or O-N(CH_3)-CH_2-CH_2 linkage alternating with a phosphorothioate or phosphodiester linkage.

2. The oligonucleotide of claim 1 wherein said at least one CH_2-NH-O-CH_2, CH_2-N(CH_3)-O-CH_2, CH_2-O-N(CH_3)-CH_2, CH_2-N(CH_3)-N(CH_3)-CH_2 or O-N(CH_3)-CH_2-CH_2 linkage is a methylene(methylimino) linkage.

3. The oligonucleotide of claim 1 comprising 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA deriving from the human H-ras gene.

4. The oligonucleotide of claim 3 specifically hybridizable with a translation initiation site or codon 12 of the human H-ras gene.

5. The oligonucleotide of claim 3 comprising SEQ ID No: 1, SEQ ID No: 2 or SEQ ID No: 3.

6. The oligonucleotide of claim 1 wherein at least one of the nucleosidic units of said oligonucleotide is modified at the 2′ position.

7. The oligonucleotide of claim 6 wherein said 2′ modification is 2′-fluoro, 2′-O-alkyl or 2′-O-substituted alkyl.

8. The oligonucleotide of claim 7 wherein said 2′ modification is 2′-substituted alkyl.

9. The oligonucleotide of claim 8 wherein said 2′ substituted alkyl is 2′-O-methoxyethyl.

10. The oligonucleotide of claim 2 wherein said methylene(methylimino) linkage is alternating with a phosphorothioate linkage.

11. The oligonucleotide of claim 2 wherein said methylene(methylimino) linkage is alternating with a phosphodiester linkage.

12. The oligonucleotide of claim 2 comprising one, two or three methylene(methylimino) linkages alternating with phosphorothioate or phosphodiester linkages.

13. The oligonucleotide of claim 12 comprising one or two methylene(methylimino) linkages alternating with phosphorothioate linkages.

14. An oligonucleotide comprising from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA and being a substrate for RNAse H and having at least one methylene(methylimino) linkage.

15. The oligonucleotide of claim 14 specifically hybridizable with DNA or mRNA deriving from the human H-ras gene.

16. The Oligonucleotide of claim 15 comprising SEQ ID No: 1, SEQ ID No: 2 and SEQ ID No: 3.

17. A chimeric oligonucleotide comprising from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA and containing a first region having at least one methylene(methylimino) linkage and a second region having at least one phosphorothioate linkage.

18. The chimeric oligonucleotide of claim 17 wherein said second region is flanked by two of said first regions each of which includes at least one methylene(methylimino) linkage.

19. The chimeric oligonucleotide of claim 17 wherein said first region includes at least one nucleosidic unit modified at the 2′ position.

20. The chimeric oligonucleotide of claim 19 wherein the modification at the 2′ position is a 2′-O-alkyl, 2′-O-substituted alkyl or a 2′-fluoro modification.

21. The chimeric oligonucleotide of claim 15 wherein said second region comprises 2′-deoxynucleotides.

22. The chimeric oligonucleotide of claim 21 wherein said second region is at least four nucleotides long.

23. The chimeric oligonucleotide of claim 21 wherein said second region is five to nine nucleotides long.

24. The chimeric oligonucleotide of claim 17 wherein said first region includes at least one methylene(methylimino) linkage alternating with a phosphorothioate or phosphodiester linkage.

25. The chimeric oligonucleotide of claim 24 wherein said first region includes one, two or three methylene(methylimino) linkages alternating with phosphorothioate or phosphodiester linkages.

26. The chimeric oligonucleotide of claim 25 wherein said first region includes one, two or three methylene(methylimino) linkages alternating with phosphorothioate linkages.

27. The chimeric oligonucleotide of claim 25 wherein said first region includes one, two or three methylene(methylimino) linkages alternating with phosphodiester linkages.

28. An oligonucleotide comprising from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA deriving from the human H-ras gene and and having at least one methylene(methylimino) internucletoside linkage.

29. The oligonucleotide of claim 28 wherein said oligonucleotide is specifically hybridizable with a translation initiation site or codon 12 of the human H-ras gene.

30. An oligonucleotide comprising from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA deriving from the human H-ras gene and and containing a first region having at least one nucleosidic unit modified at the 2′ position with a 2′-O-methoxyethyl substituent and a second region having at least one phosphorothioate linkage.

31. The oligonucleotide of claim 30 wherein said first region has at least three nucleosidic units modified at the 2′ position with 2′-O-methoxyethyl substituents.

32. The oligonucleotide of claim 30 wherein said second region has at least four nucleosidic units linked together with phosphorothioate linkages.

33. The oligonucleotide of claim 31 wherein each of the nucleosidic units of said second region are 2′-deoxynucleotides.

34. The chimeric oligonucleotide of claim 33 wherein said second region is flanked by two of said first regions each

of which includes at least one nucleosidic unit modified at the 2′ position with a 2′-O-methoxyethyl substituent.

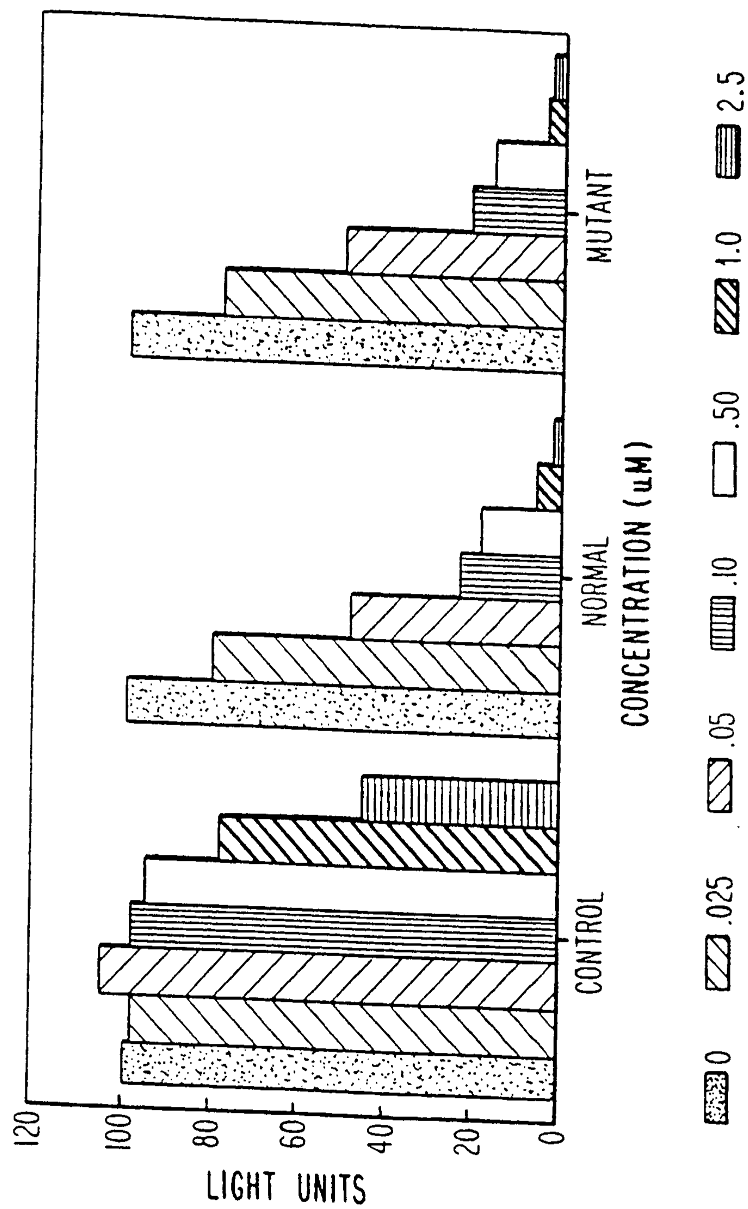
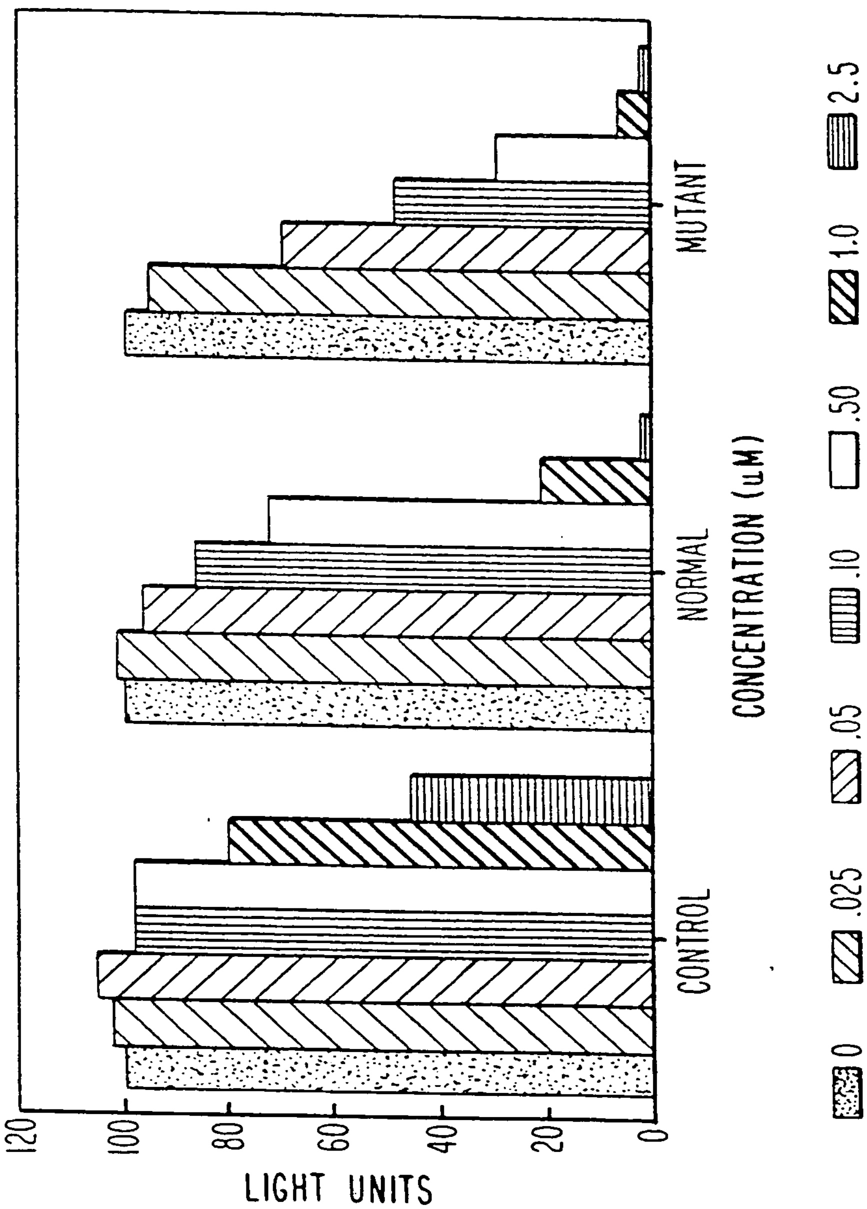

LIGHT UNITS
0
20
40
60
80
100
120
CONTROL
NORMAL
MUTANT
CONCENTRATION (uM)
0
.025
.05
.10
.50
1.0
2.5

Fig. 1


LIGHT UNITS
120
100
80
60
40
20
0
CONTROL
NORMAL
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CONCENTRATION (uM)
0
.025
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1.0
2.5

Fig. 2

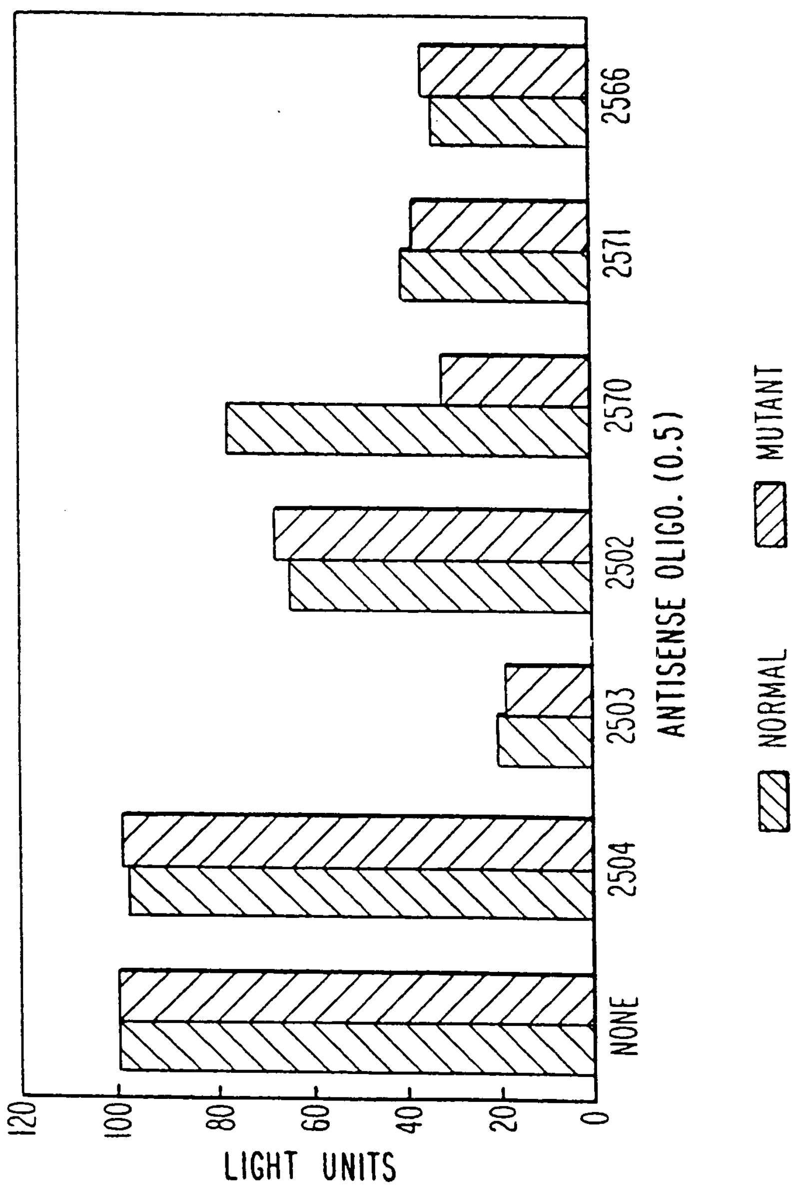
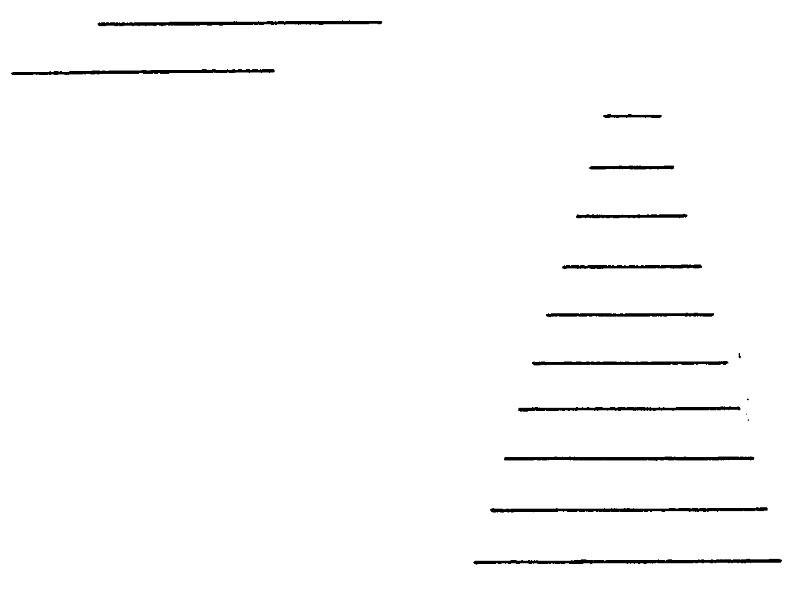

LIGHT UNITS
120
100
80
60
40
20
0
NONE
2504
2503
2502
2570
2571
2566
ANTISENSE OLIGO. (0.5)
NORMAL
MUTANT

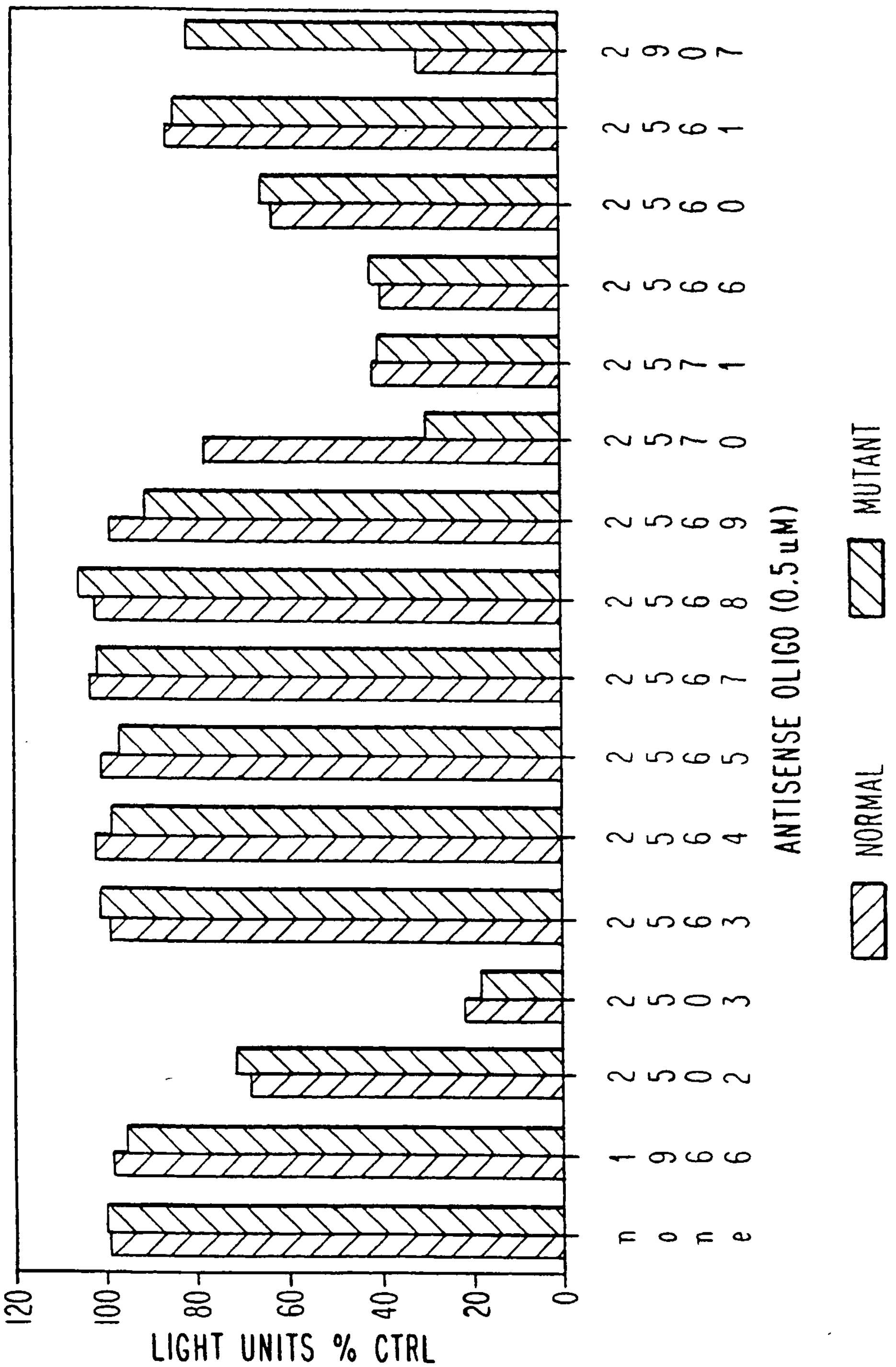
Fig. 3

ggccccugaggagcgAUGacggaauauaagcugguggugguggggcgccgUcggugugggcaagagugcgcug



OLIGO	LENGTH	TARGET	IC50 (uM)	SELECTIVITY
2502	20	AUG	0.75	NONE
2503	20	AUG	0.05	NONE
2563	5	POINT	NOT ACTIVE	----
2564	7	POINT	NOT ACTIVE	----
2565	9	POINT	NOT ACTIVE	----
2567	11	POINT	NOT ACTIVE	----
2568	13	POINT	NOT ACTIVE	----
2569	15	POINT	NOT ACTIVE	----
2570	17	POINT	0.10	2-3x
2571	19	POINT	0.25	NONE
2566	21	POINT	0.25	NONE
2560	23	POINT	0.75	NONE
2561	25	POINT	1.00	NONE

Fig. 4a



LIGHT UNITS % CTRL
120
100
80
60
40
20
0
ANTISENSE OLIGO (0.5 uM)
none
1966
2502
2503
2563
2564
2565
2567
2568
2569
2570
2571
2566
2560
2561
2907
NORMAL
MUTANT

Fig. 4b

Sequence	OLIGO	LENGTH	TARGET
ggccccugaggagcgAUGacggaauauaagcugguggugguggggcgccg(G↓)Ucggugugggcaagagugcgcug			
ctcgctactgccttatattc	2502	20	AUG
gggactcctcgctactgcct	2503	20	AUG
gcagc	2563	5	CODON 12
ggcagcc	2564	7	CODON 12
cggcagcca	2565	9	CODON 12
gcggcagccac	2567	11	CODON 12
cgcggcagccaca	2568	13	CODON 12
ccgcggcagccacac	2569	15	CODON 12
ccgcggcagccacacc	3426	16	CODON 12
cccgcggcagccacac	3427	16	CODON 12
cccgcggcagccacacc	2570	17	CODON 12
cccgcggcagccacaccc	3428	18	CODON 12
acccgcggcagccacacc	3429	18	CODON 12
acccgcggcagccacaccc	2571	19	CODON 12
cacccgcggcagccacacccg	2566	21	CODON 12
ccacccgcggcagccacacccgt	2560	23	CODON 12
accacccgcggcagccacacccgtt	2561	25	CODON 12
cccgcggccgccacacc	2907	17	CODON 12 (wild type)

Fig. 5A

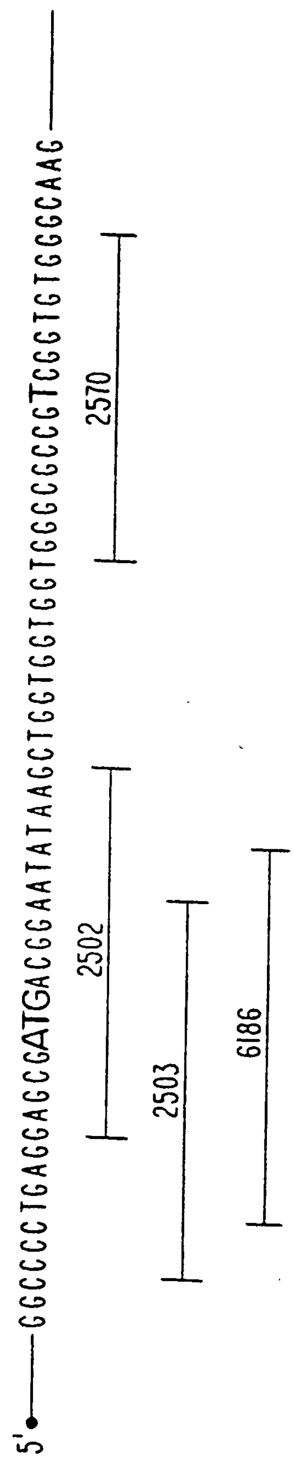

5'
GGCCCCTGAGGAGCCGATGACGGAATATAAGCTGGTGGTGGTGGGCGCCGTCGGTGTGGGCAAG
2570
2502
2503
6186

Fig. 5B

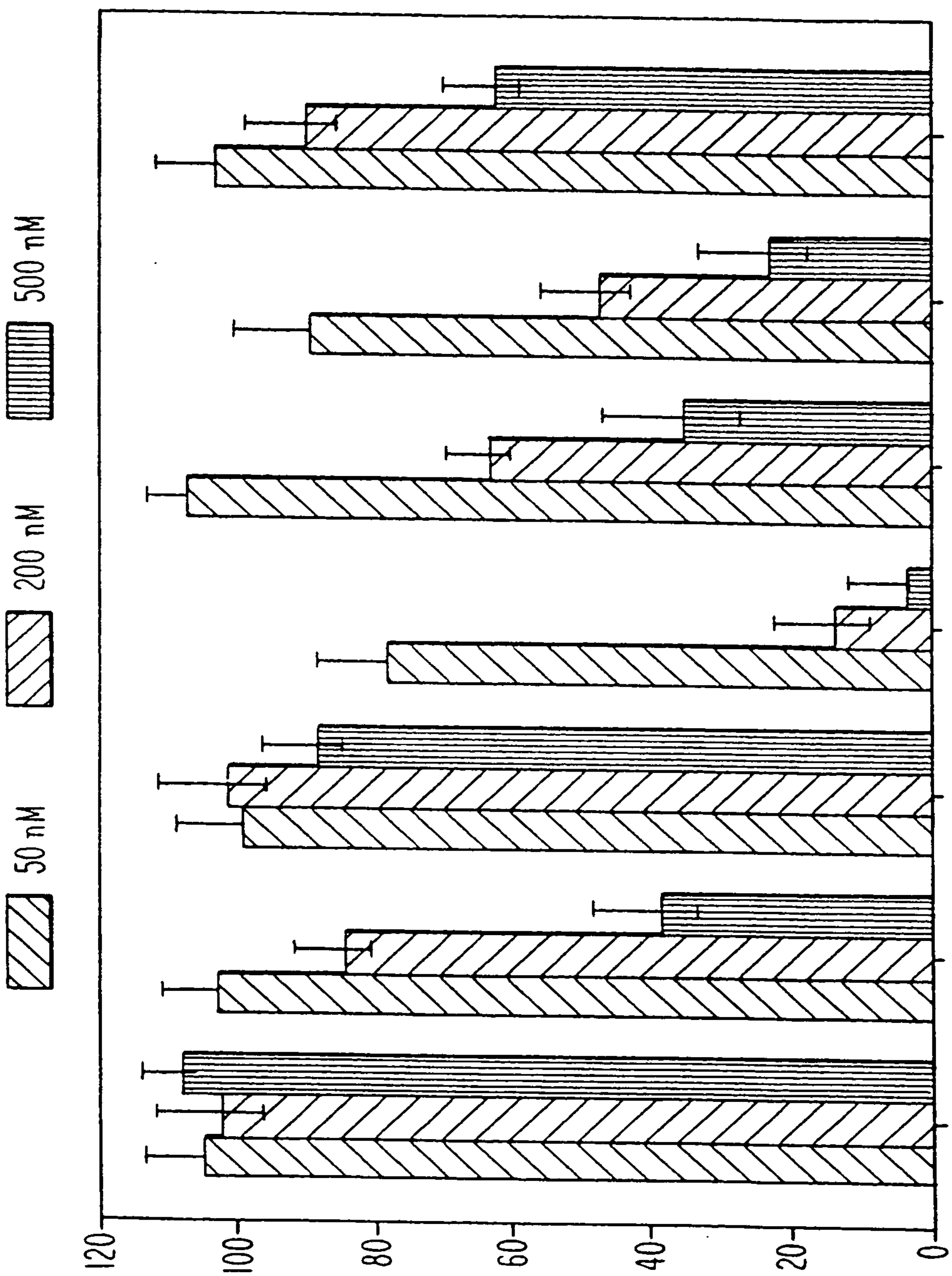

50 nM
200 nM
500 nM
120
100
80
60
40
20
0
CONTROL
2502
2502 OMe
2503
2503 OMe
6186
6186 OMe

Fig. 6

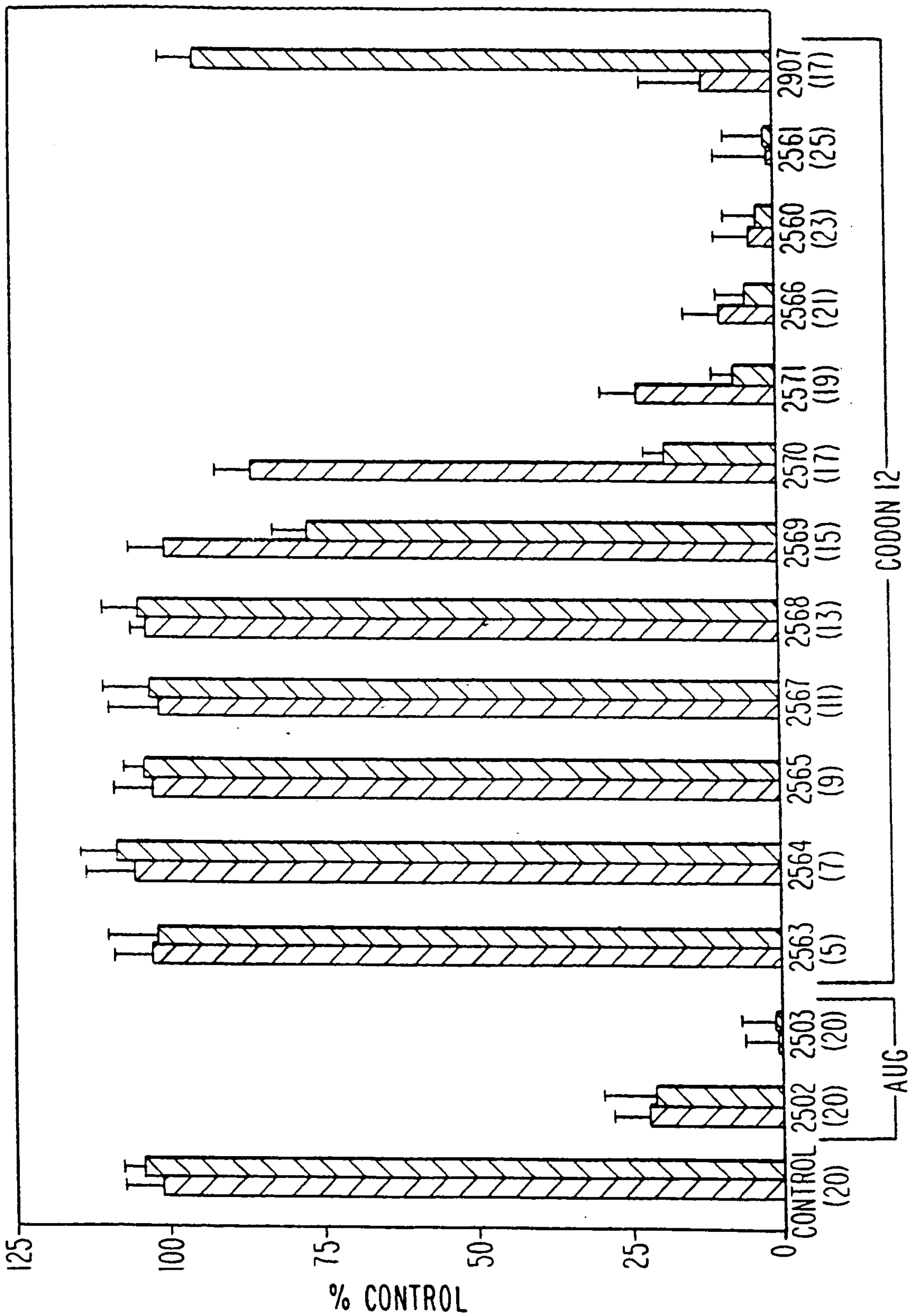

% CONTROL
0
25
50
75
100
125
CONTROL (20)
2502 (20)
2503 (20)
AUG
2563 (5)
2564 (7)
2565 (9)
2567 (11)
2568 (13)
2569 (15)
2570 (17)
2571 (19)
2566 (21)
2560 (23)
2561 (25)
2907 (17)
CODON 12

Fig. 7

Fig. 8a

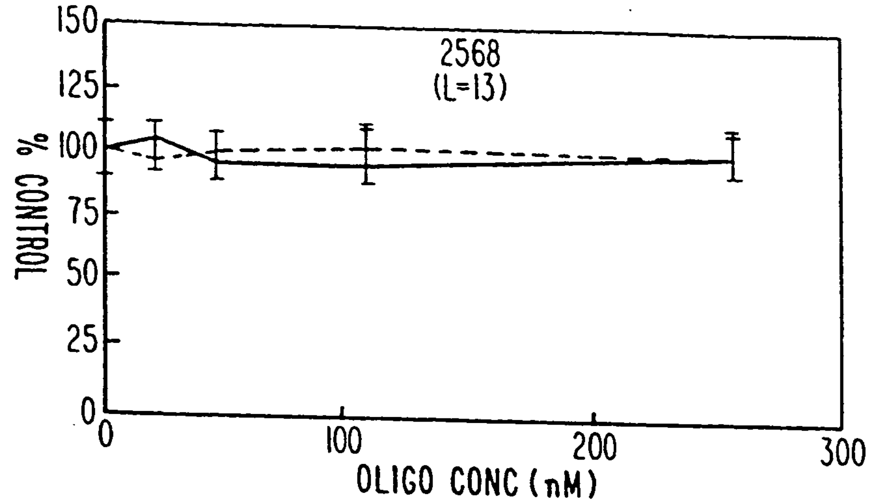


Fig. 8b

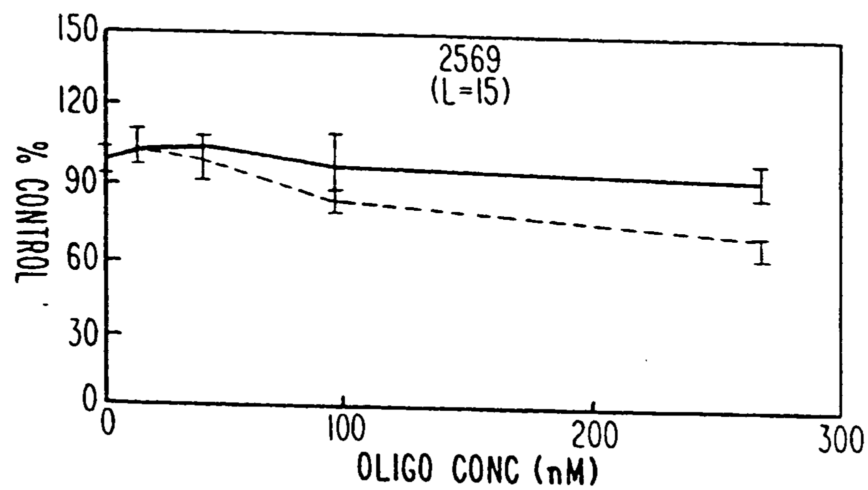


Fig. 8c

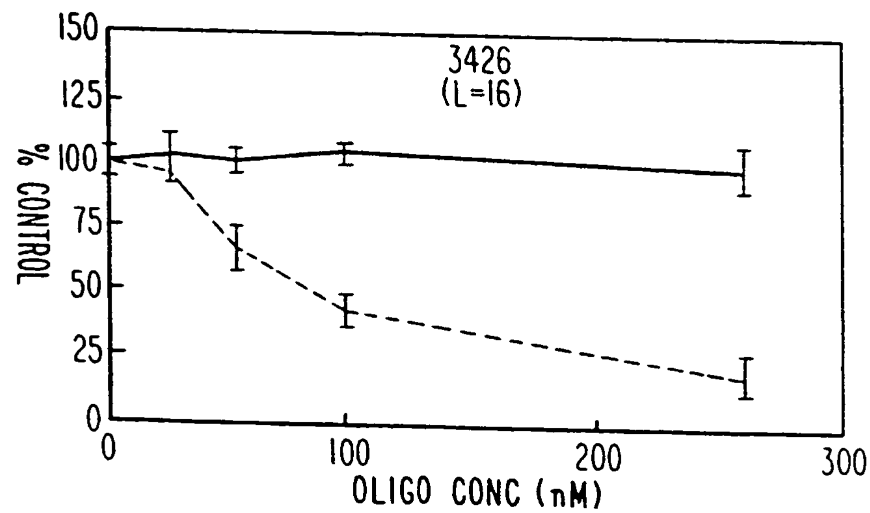


Fig. 8d

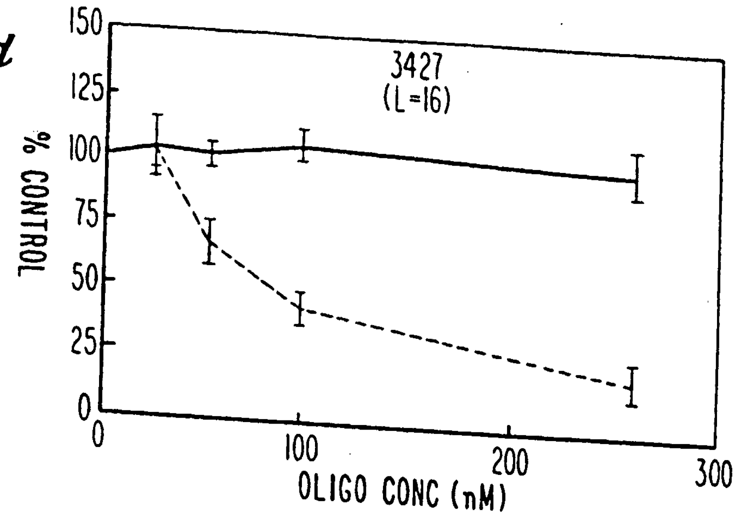


Fig. 8e

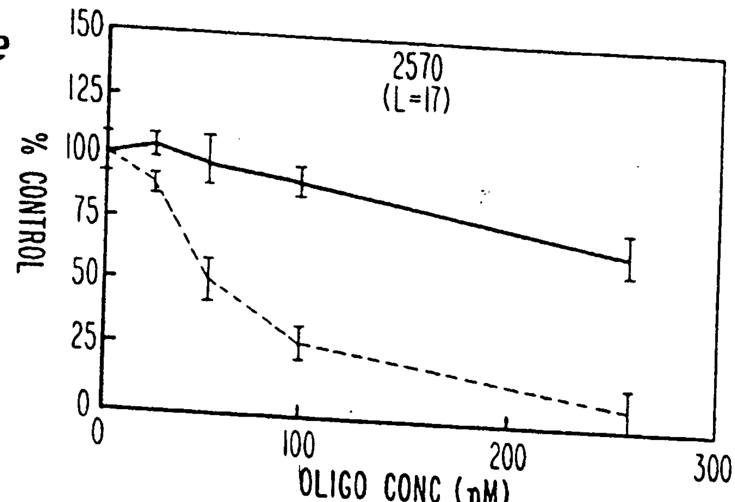
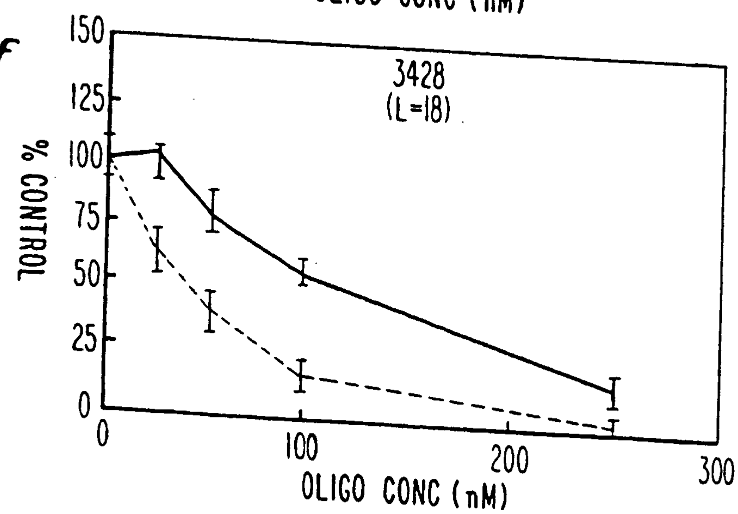


Fig. 8f



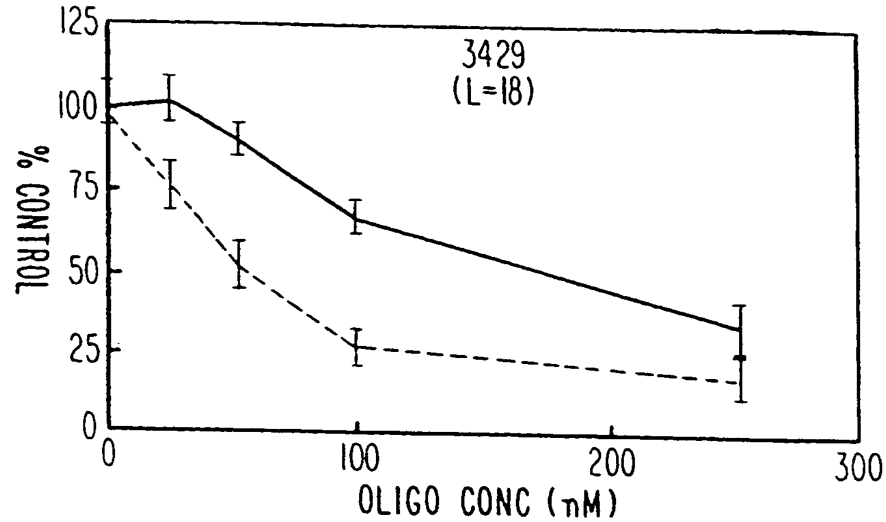

3429
(L=18)
125
100
75
50
25
0
% CONTROL
0
100
200
300
OLIGO CONC (nM)

Fig. 8g

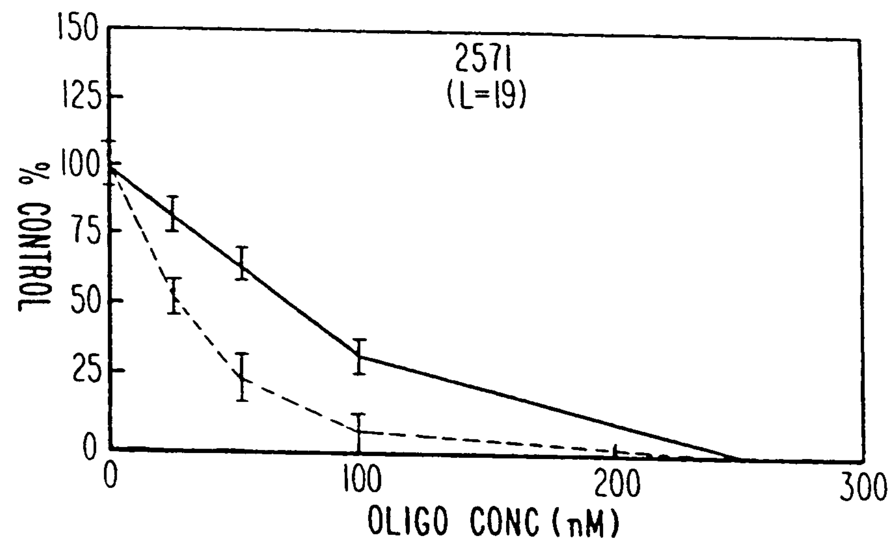

2571
(L=19)
150
125
100
75
50
25
0
% CONTROL
0
100
200
300
OLIGO CONC (nM)

Fig. 8h

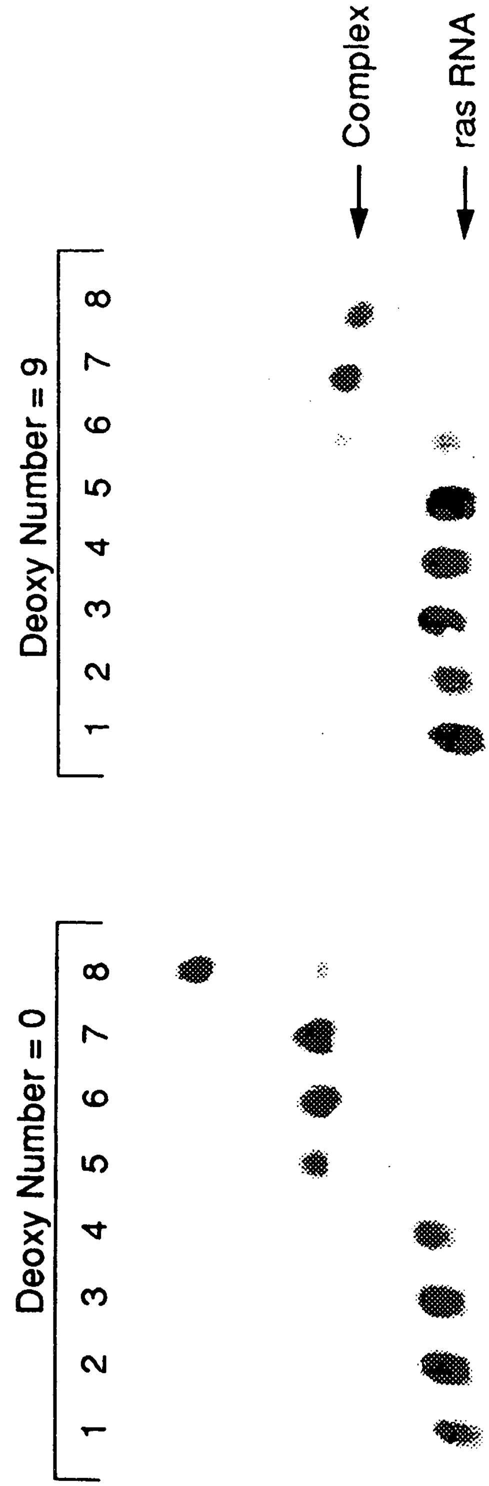
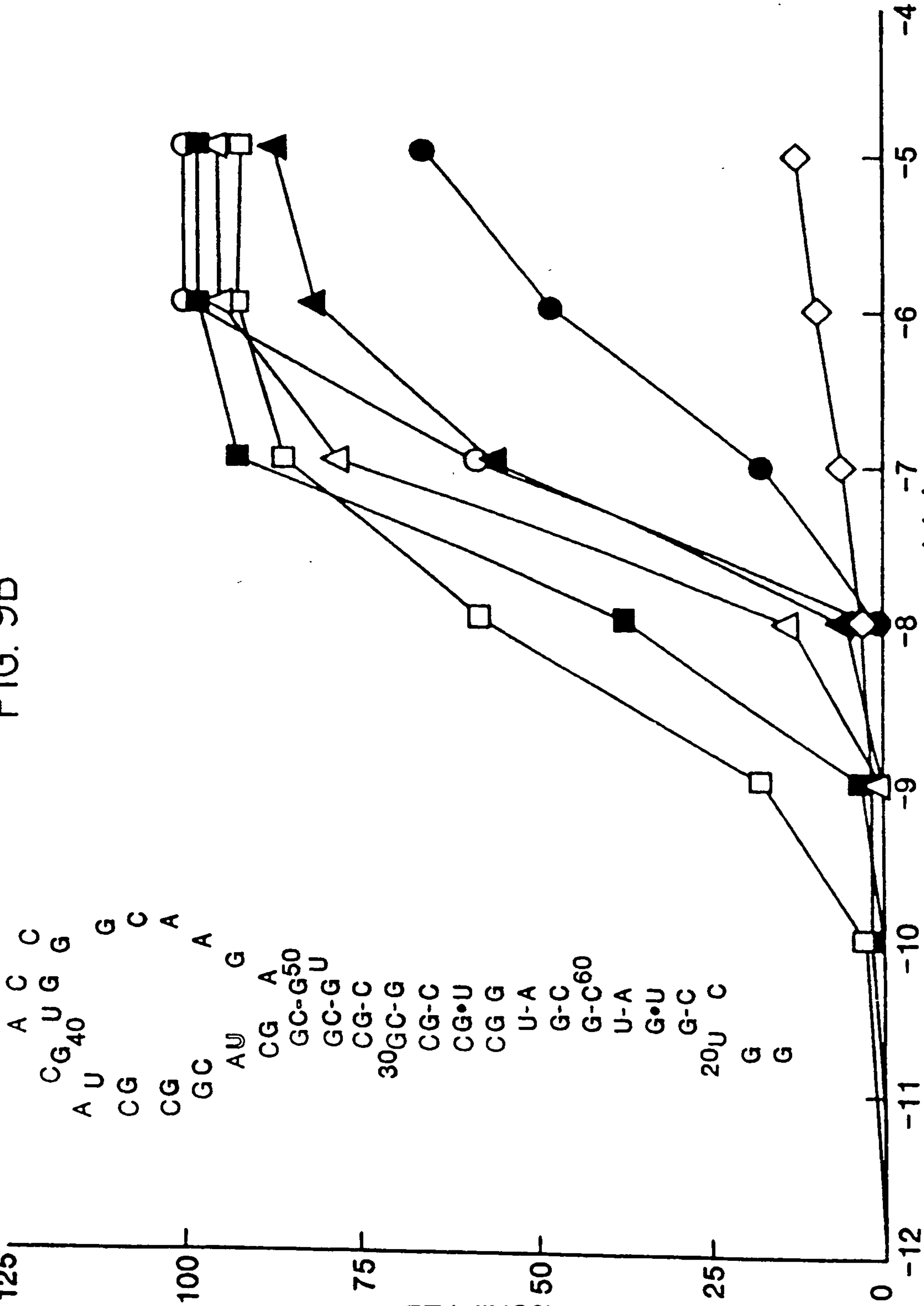

Deoxy Number = 9
1 2 3 4 5 6 7 8
Complex
ras RNA
Deoxy Number = 0
1 2 3 4 5 6 7 8

FIG.9A

FIG. 9B


%SHIFTED
0
25
50
75
100
125
log(OLIGONUCLEOTIDE) (M)
-12
-11
-10
-9
-8
-7
-6
-5
-4

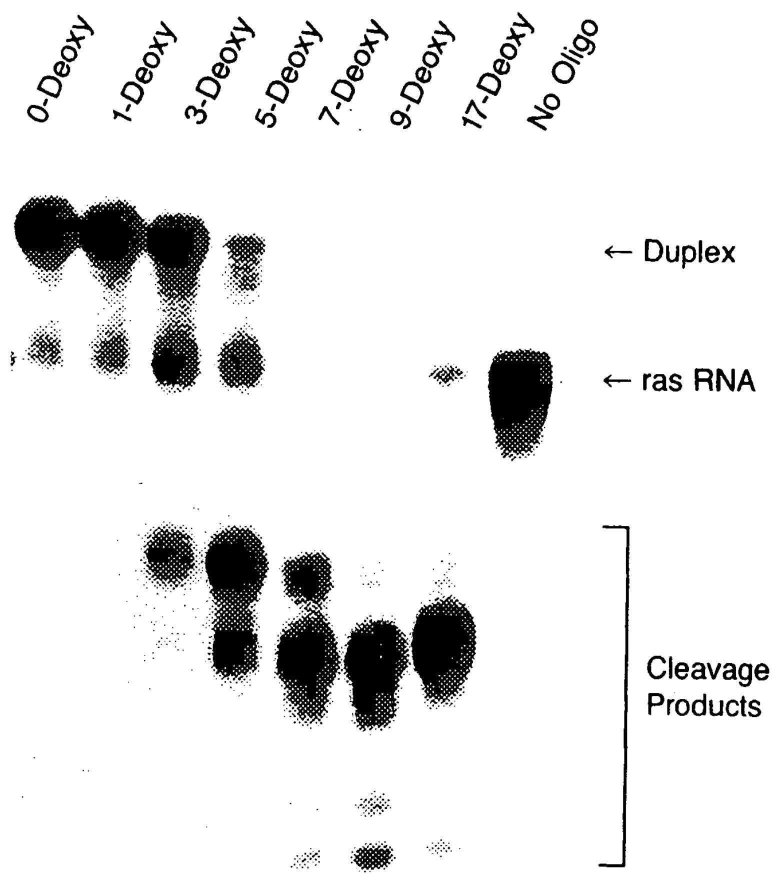

0-Deoxy
1-Deoxy
3-Deoxy
5-Deoxy
7-Deoxy
9-Deoxy
17-Deoxy
No Oligo
← Duplex
← ras RNA
Cleavage Products

FIG.10

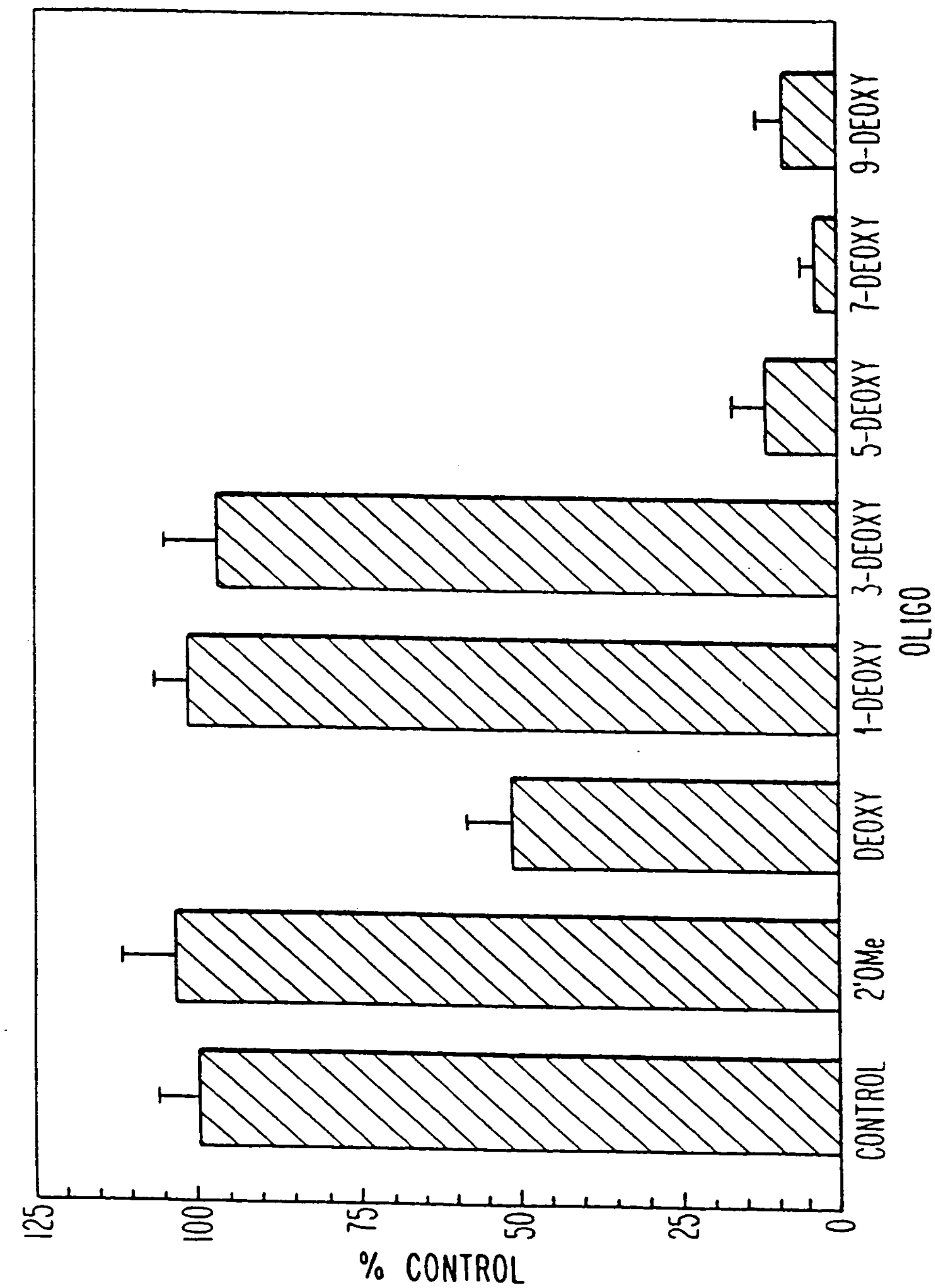

CONTROL
2'OMe
DEOXY
1-DEOXY
3-DEOXY
5-DEOXY
7-DEOXY
9-DEOXY
OLIGO
125
100
75
50
25
0
% CONTROL

Fig. 11A

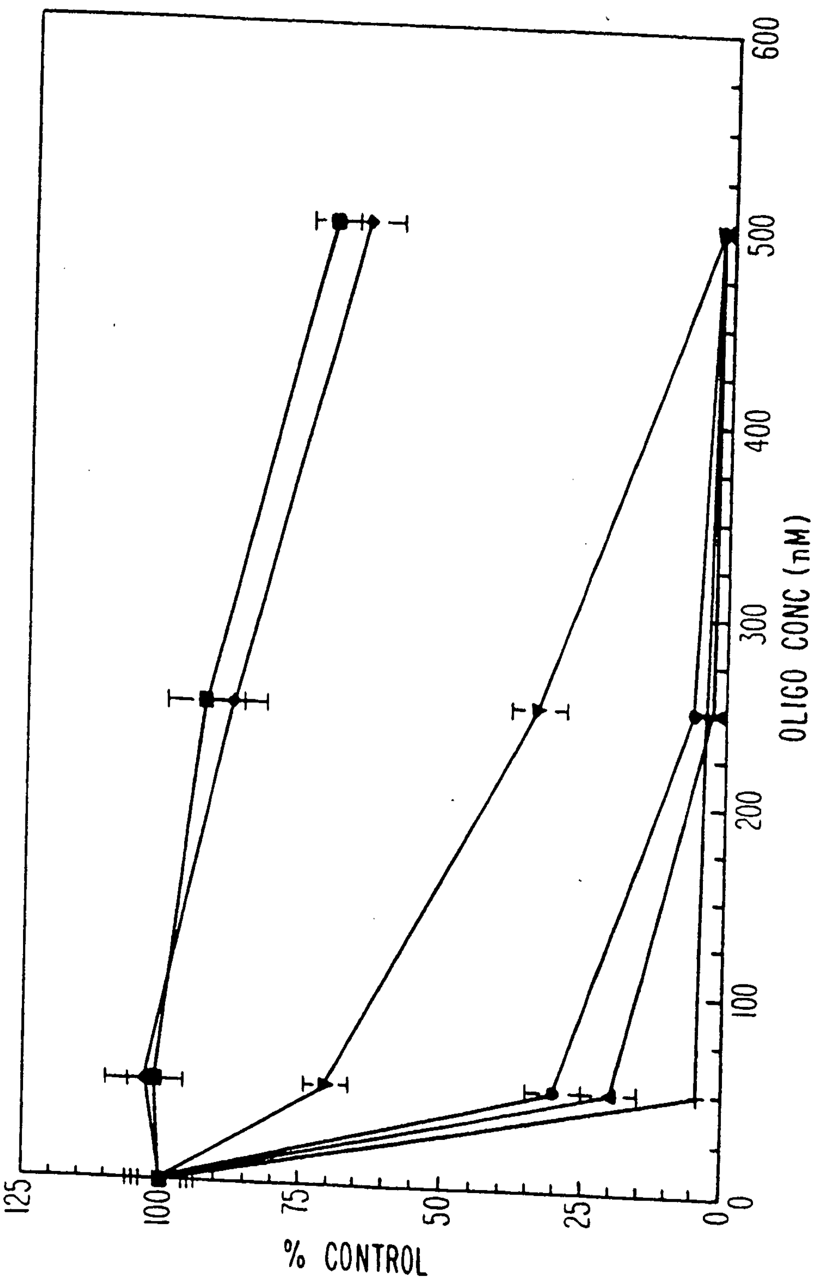

% CONTROL
125
100
75
50
25
0
OLIGO CONC (nM)
0
100
200
300
400
500
600

Fig. 11B

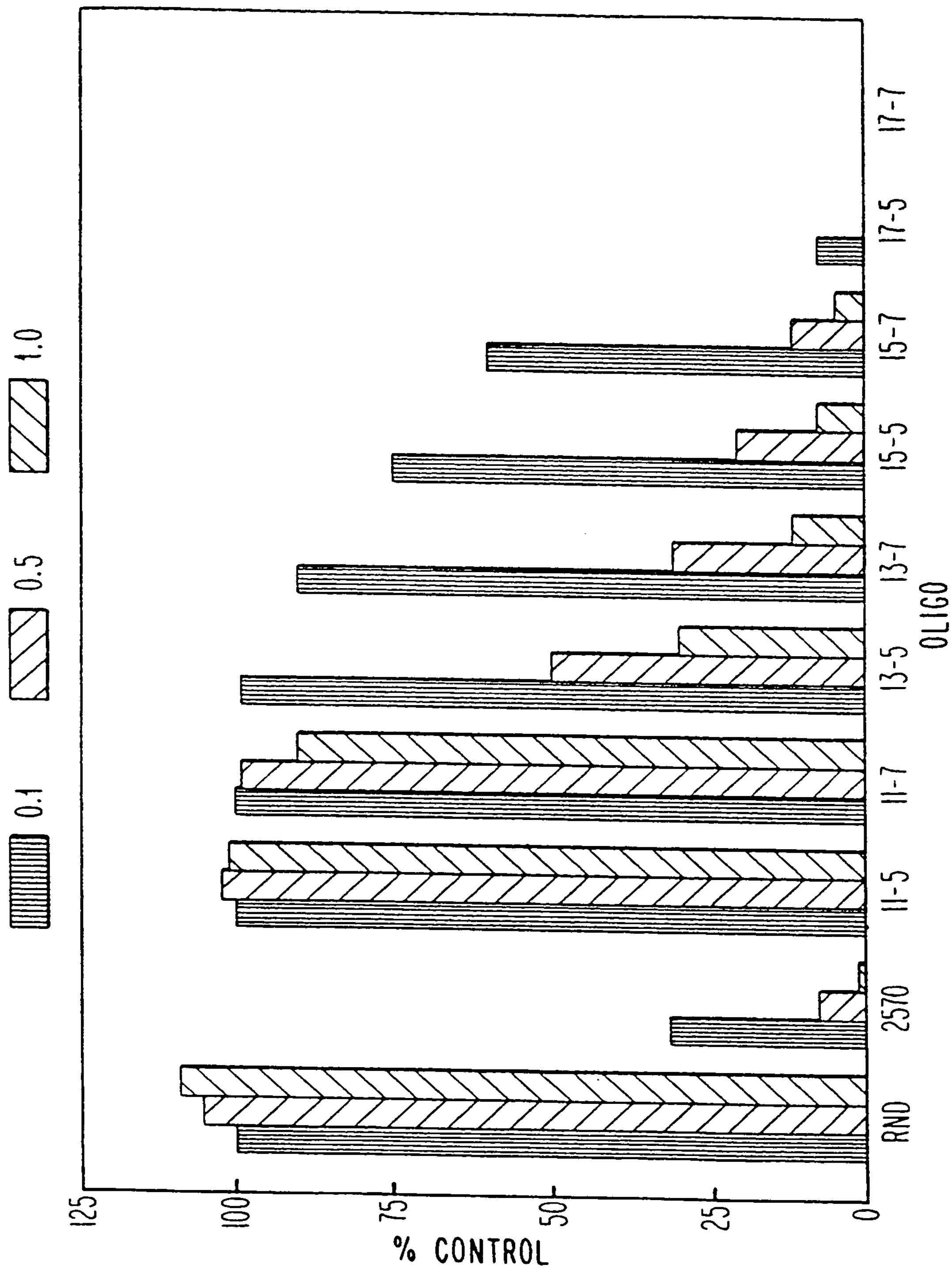

0.1
0.5
1.0
125
100
75
50
25
0
% CONTROL
RND
2570
11-5
11-7
13-5
13-7
15-5
15-7
17-5
17-7
OLIGO

Fig. 12

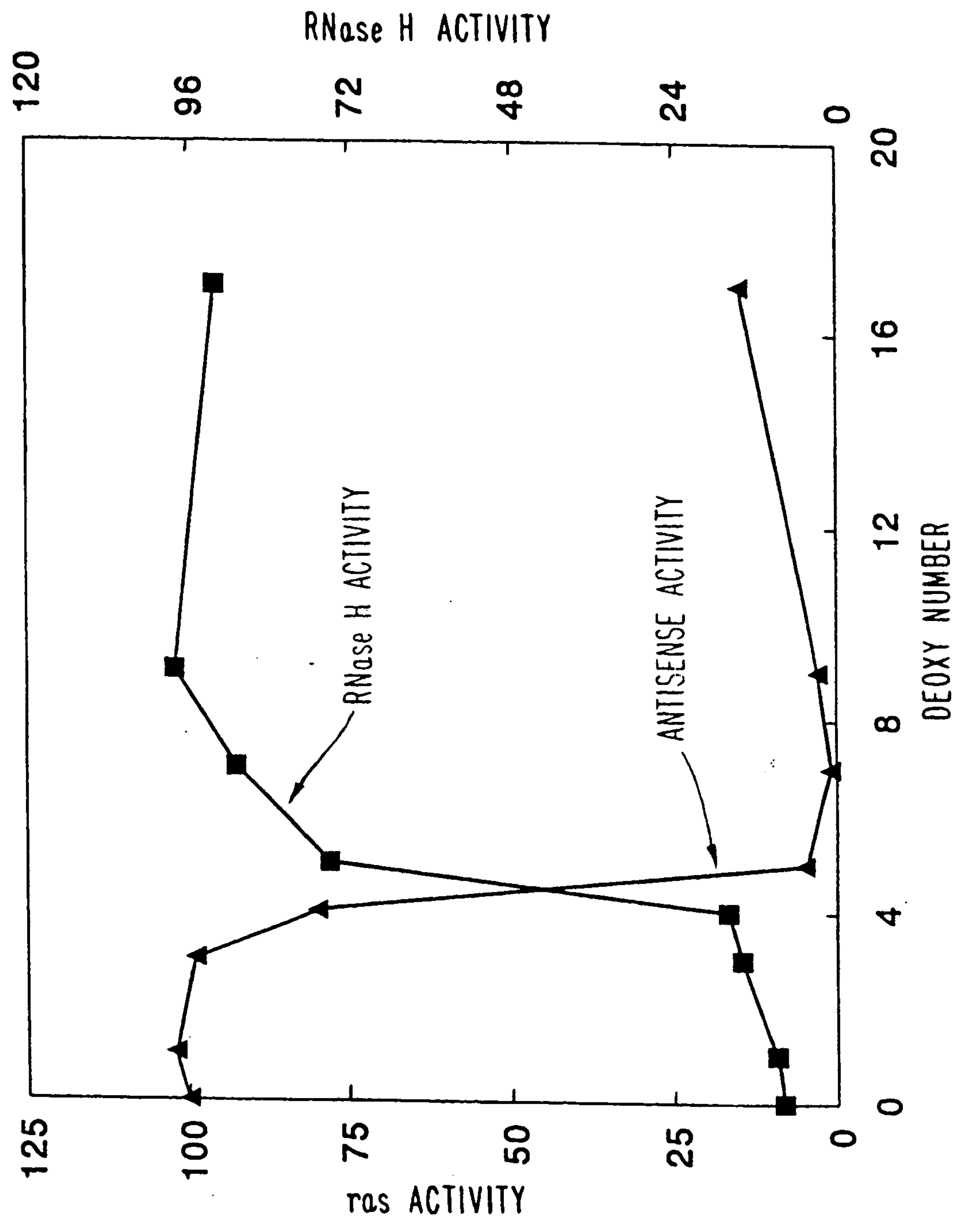
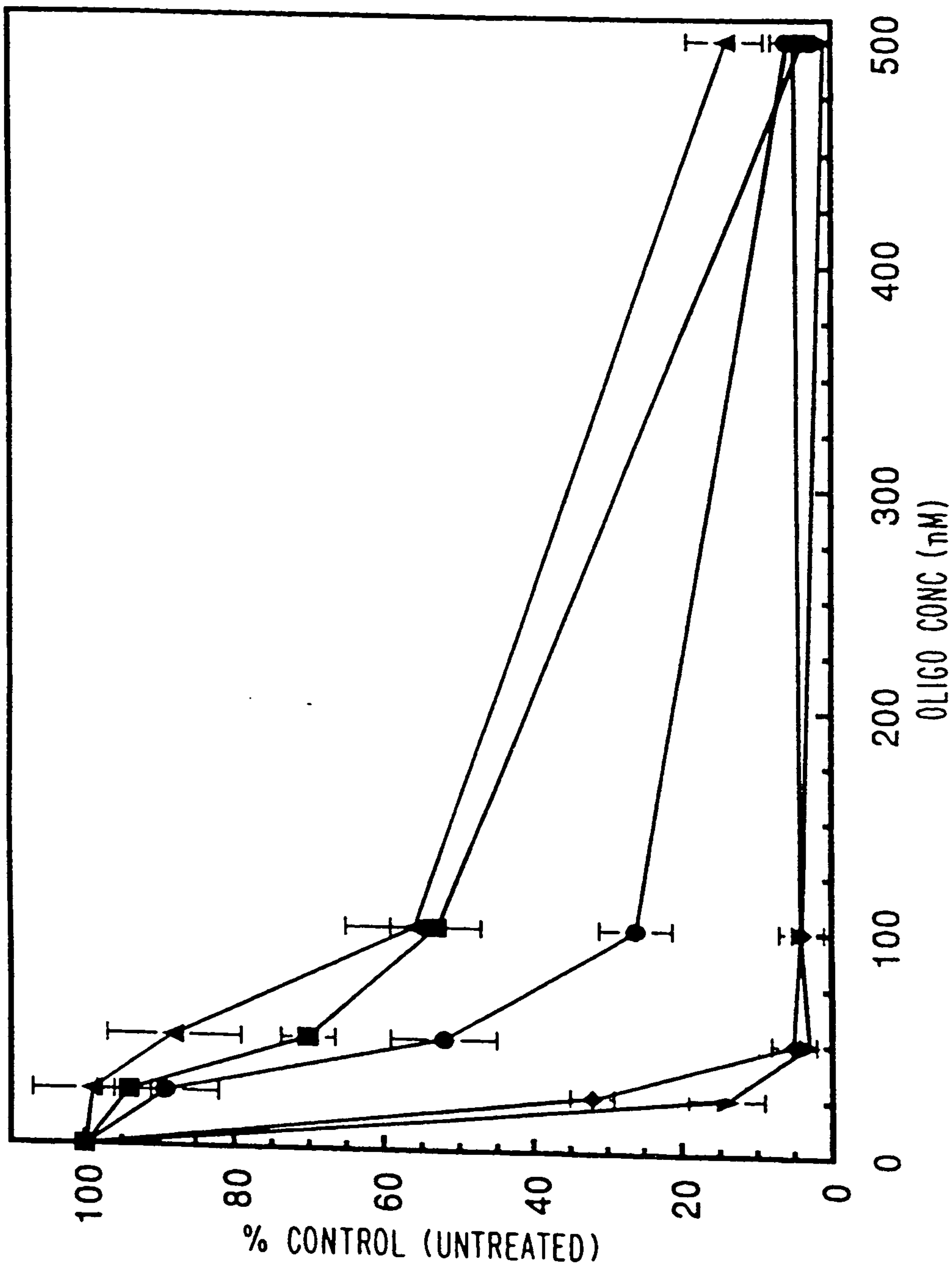

RNase H ACTIVITY
120
96
72
48
24
0
20
16
12
8
4
0
RNase H ACTIVITY
ANTISENSE ACTIVITY
DEOXY NUMBER
125
100
75
50
25
0
ras ACTIVITY

Fig. 13



% CONTROL (UNTREATED)
0
20
40
60
80
100
OLIGO CONC (nM)
0
100
200
300
400
500

Fig. 14

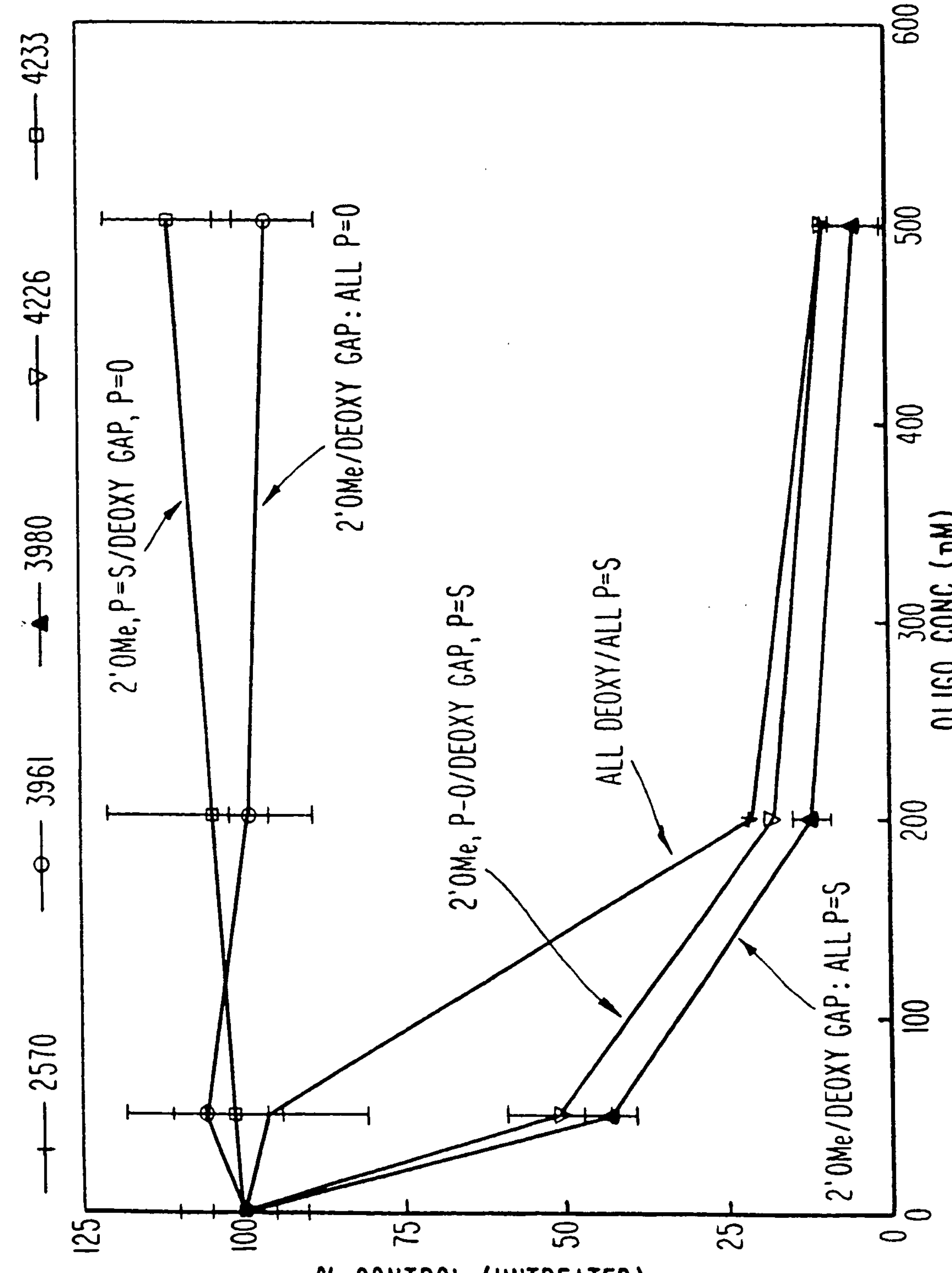


Fig. 15

RECTIFIED SHEET (RULE 91)

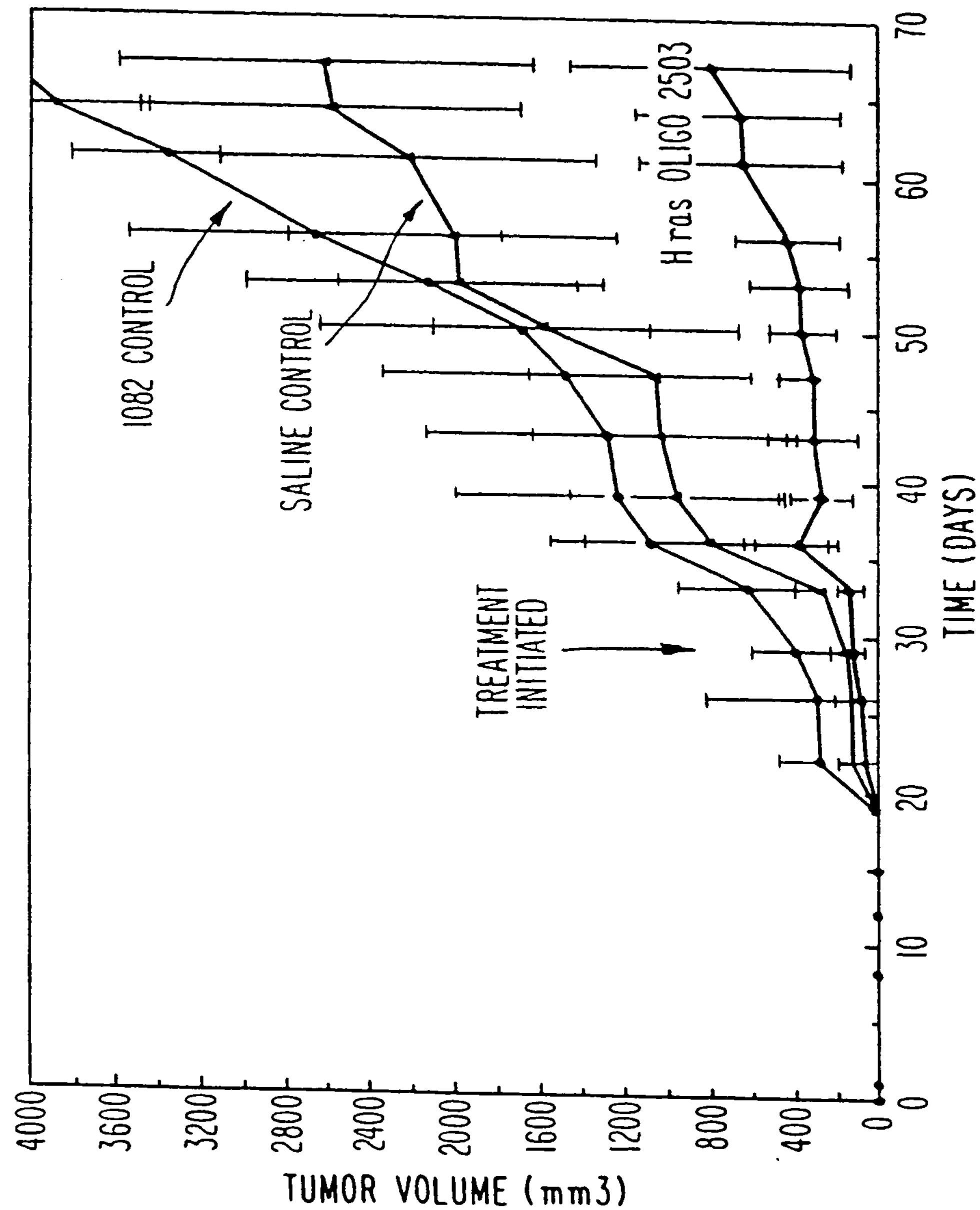

1082 CONTROL
SALINE CONTROL
Hras OLIGO 2503
TREATMENT INITIATED
TUMOR VOLUME (mm3)
TIME (DAYS)
0
400
800
1200
1600
2000
2400
2800
3200
3600
4000
0
10
20
30
40
50
60
70

Fig. 16

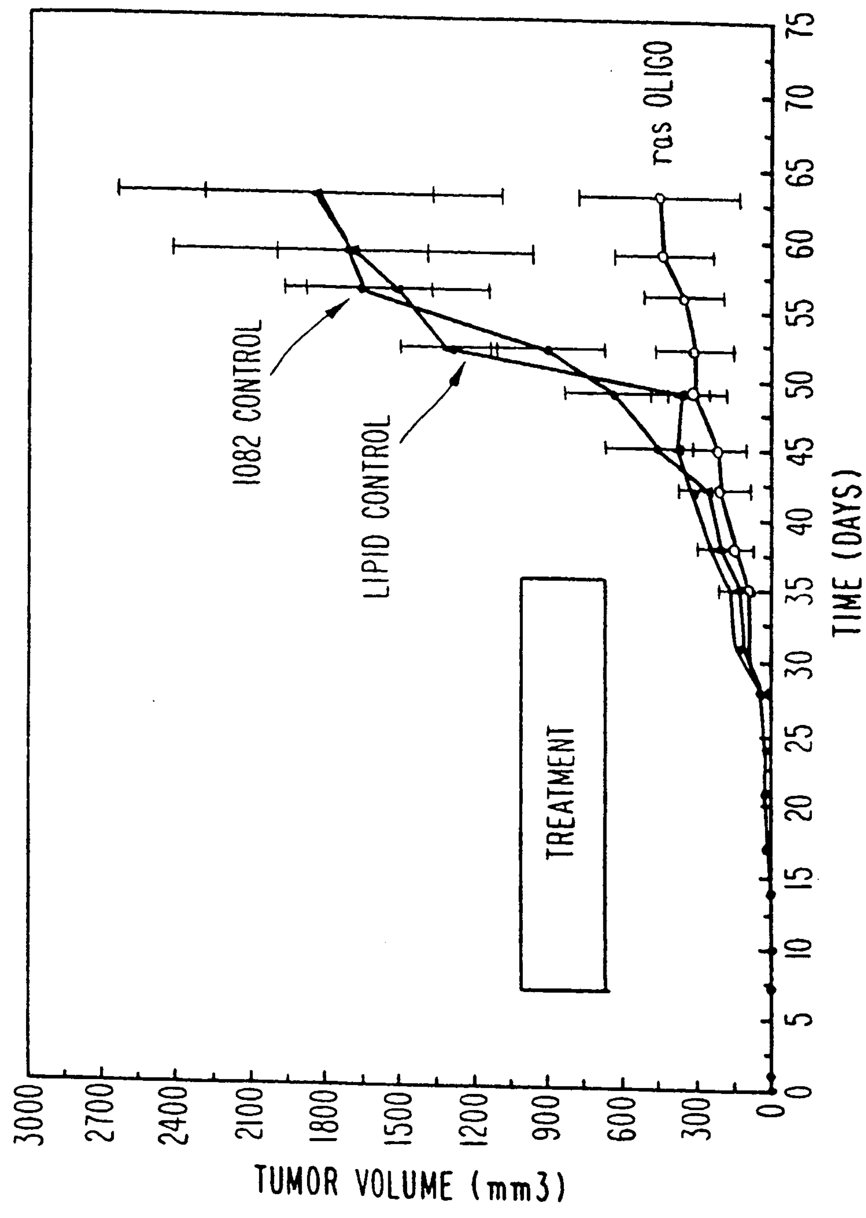

TUMOR VOLUME (mm3)
3000
2700
2400
2100
1800
1500
1200
900
600
300
0
TIME (DAYS)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
1082 CONTROL
LIPID CONTROL
ras OLIGO
TREATMENT

Fig. 17

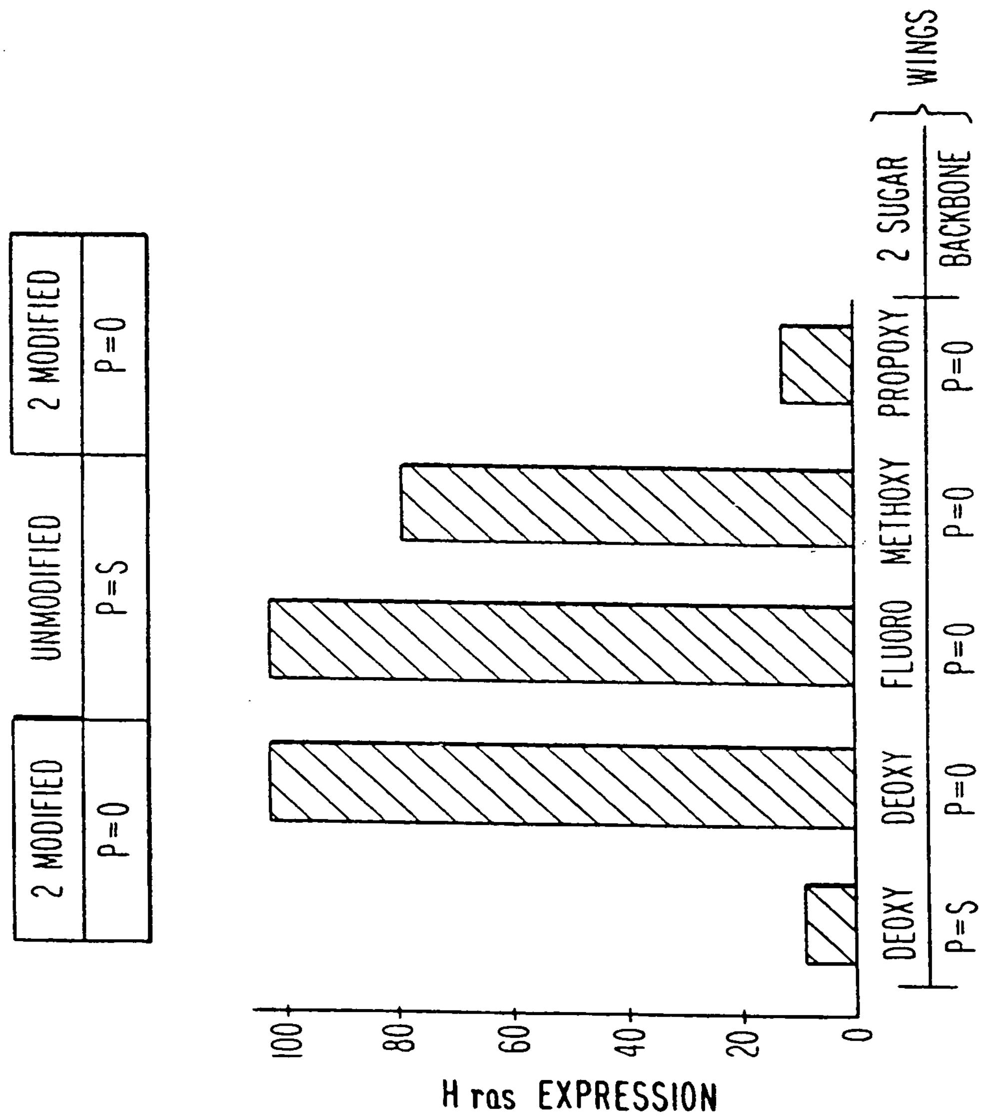

2 MODIFIED
P=0
UNMODIFIED
P=S
2 MODIFIED
P=0
H ras EXPRESSION
0
20
40
60
80
100
DEOXY
P=S
DEOXY
P=0
FLUORO
P=0
METHOXY
P=0
PROPOXY
P=0
2 SUGAR
BACKBONE
WINGS

Fig. 18

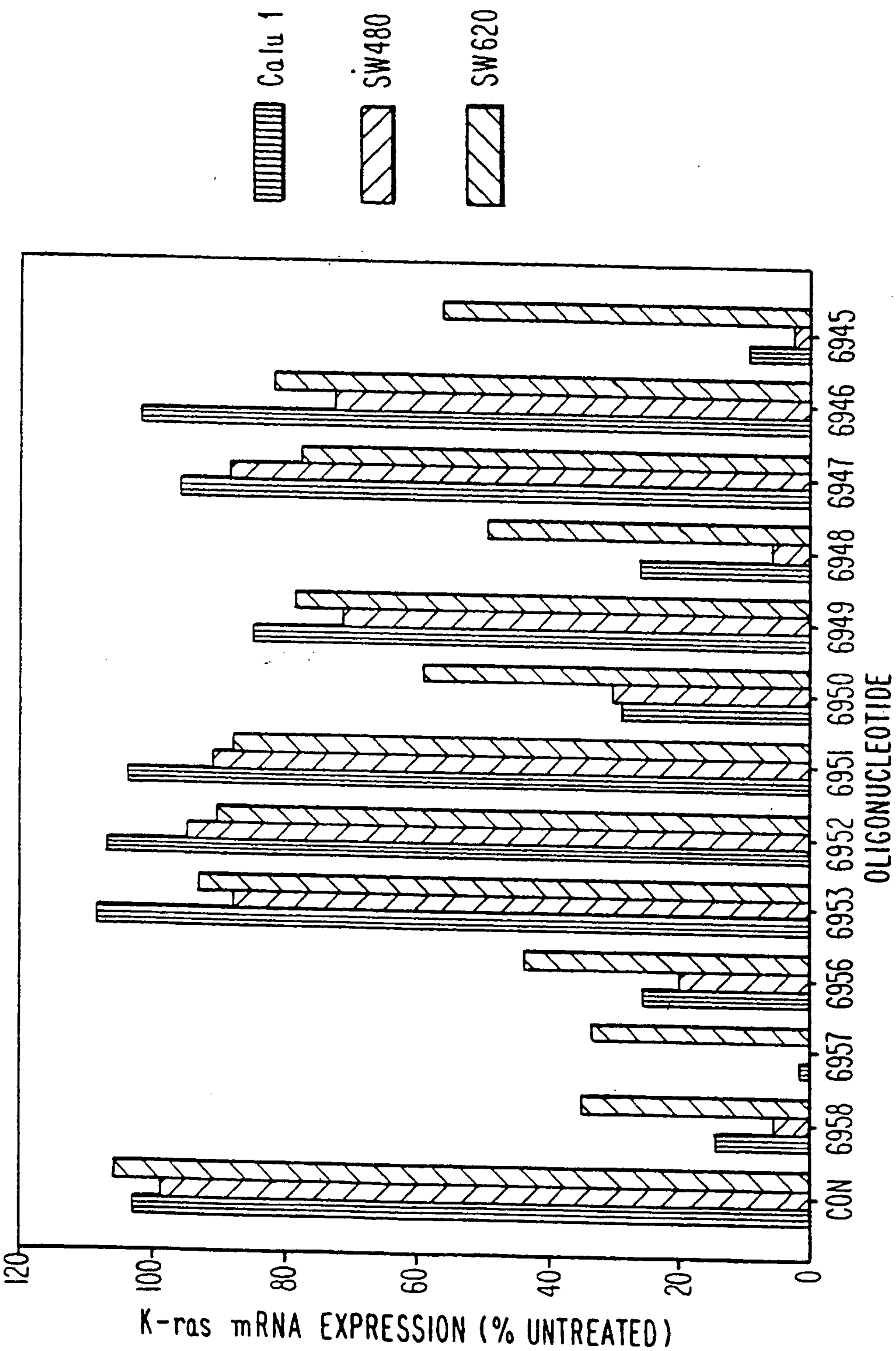

Calu 1
SW480
SW620
CON 6958 6957 6956 6953 6952 6951 6950 6949 6948 6947 6946 6945
OLIGONUCLEOTIDE
0 20 40 60 80 100 120
K-ras mRNA EXPRESSION (% UNTREATED)

Fig. 19

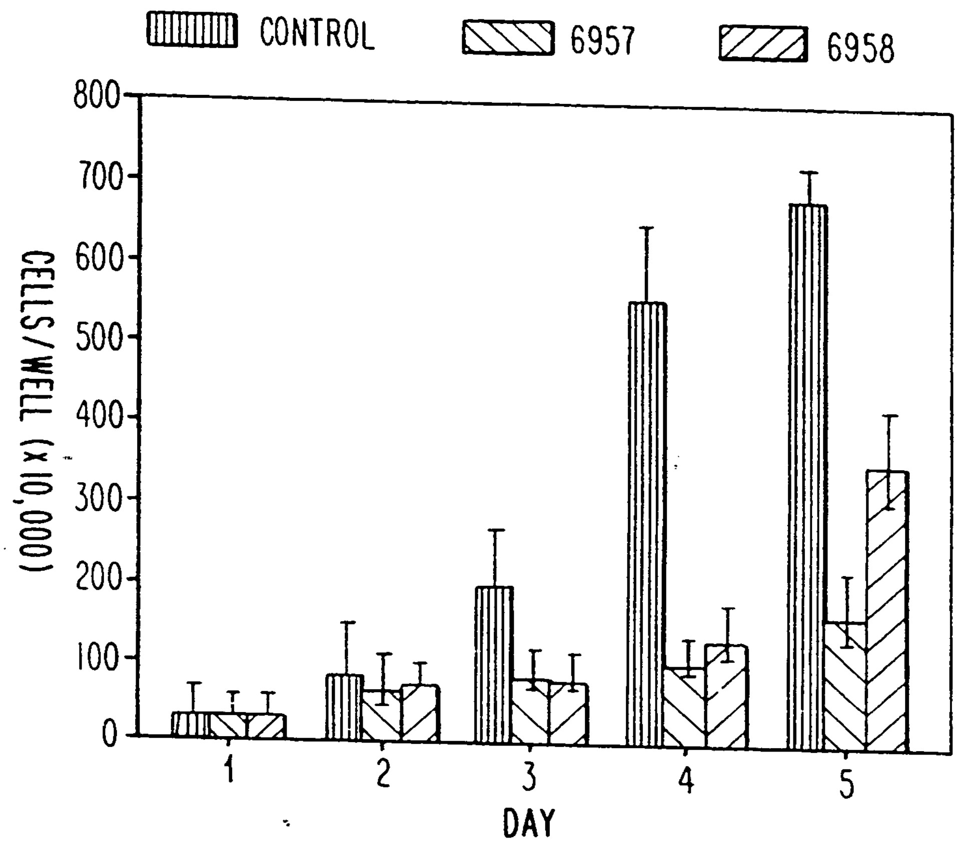

CONTROL
6957
6958
800
700
600
500
400
300
200
100
0
CELLS/WELL (x10,000)
1
2
3
4
5
DAY

Fig. 20

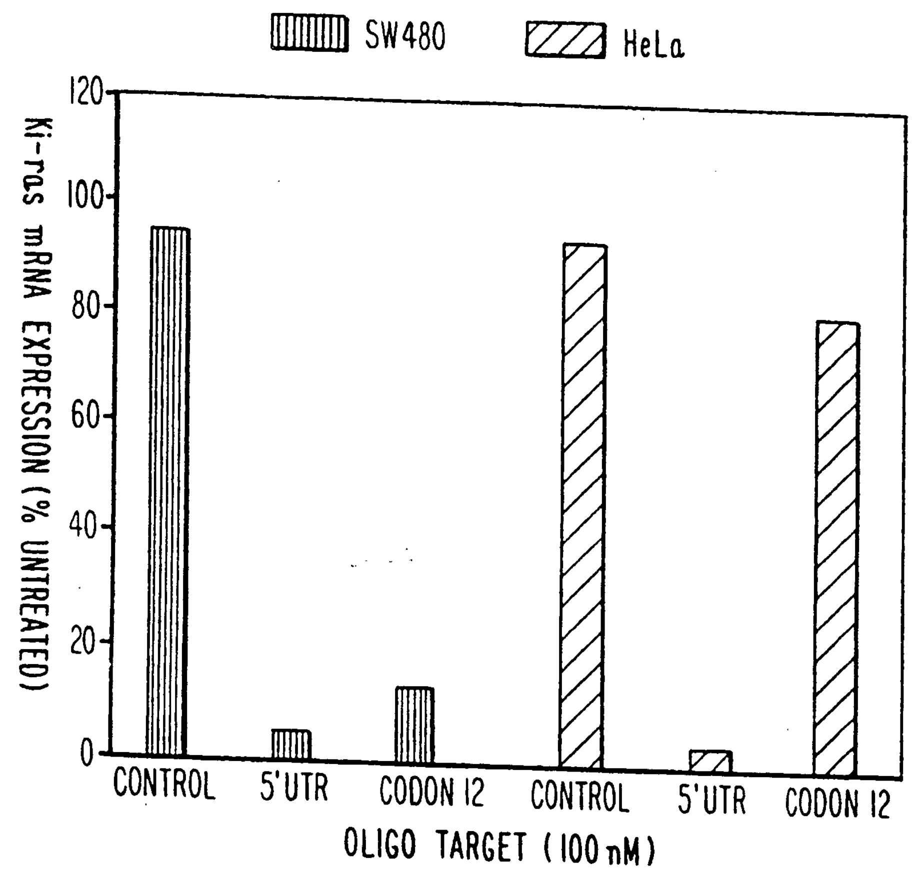

SW480
HeLa
120
100
80
60
40
20
0
Ki-ras mRNA EXPRESSION (% UNTREATED)
CONTROL
5'UTR
CODON 12
CONTROL
5'UTR
CODON 12
OLIGO TARGET (100 nM)

Fig. 21

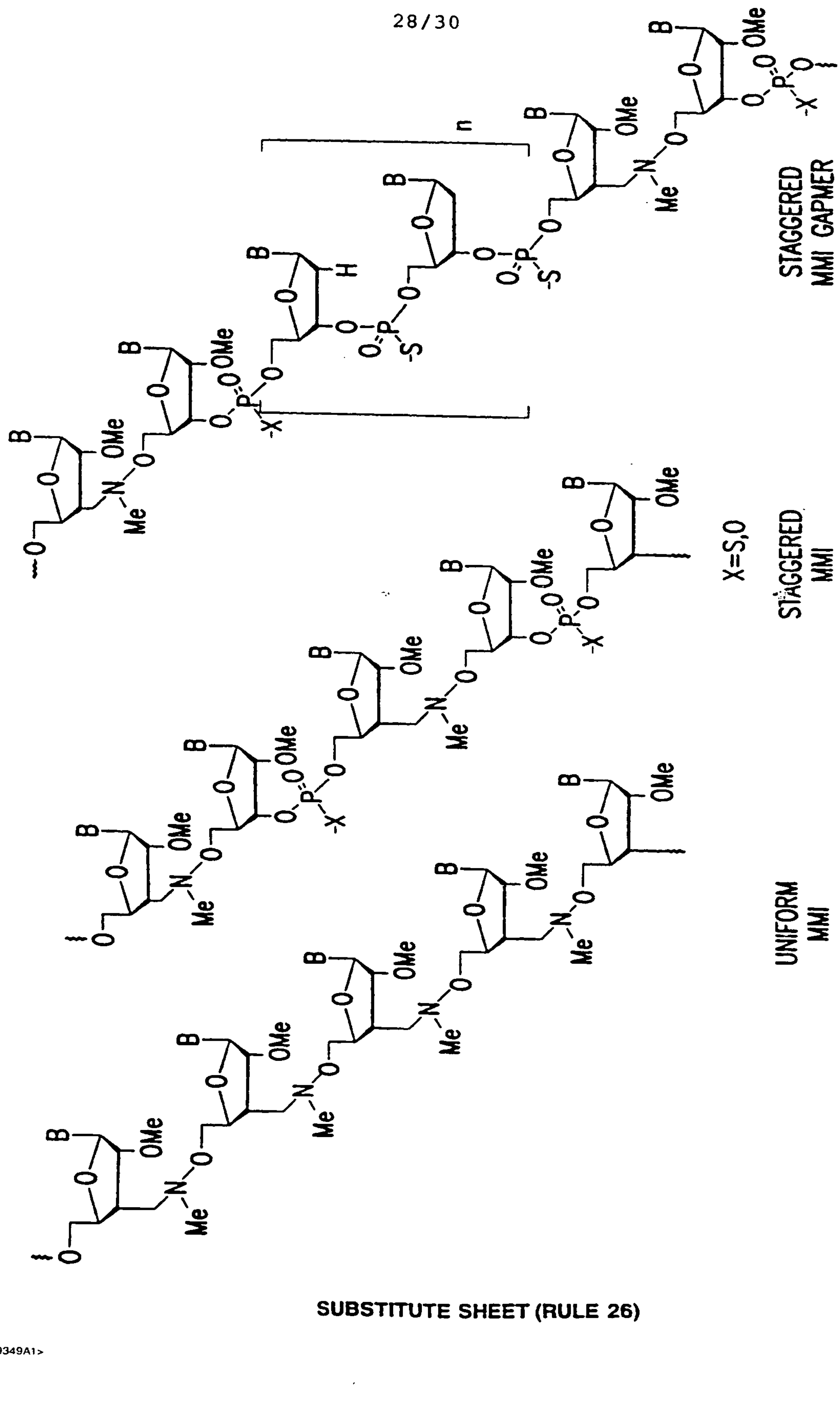


FIG. 22

ISIS 2503	TCCGTCATCGCTCCTCAGGG	Positive control, P=S
ISIS 13177	TTAGTAATAGCCCCACATGG	Scrambled control, P=S
ISIS 14896	TCCGTCATCGCTCCTCAGGG	MMI (1+1), P=S
ISIS 14898	TCCGTCATCGCTCCTCAGGG	MMI (2+2), P=S
ISIS 14890	TCCGTCATCGCTCCTCAGGG	MMI (3+3), P=S
ISIS 14897	TCCGTCATCGCTCCTCAGGG	MMI (2+2), P=O
ISIS 14899	TCCGTCATCGCTCCTCAGGG	MMI (3+3), P=O

P=S =phosphorothioate; P=O =phosphodiester

FIG. 23A

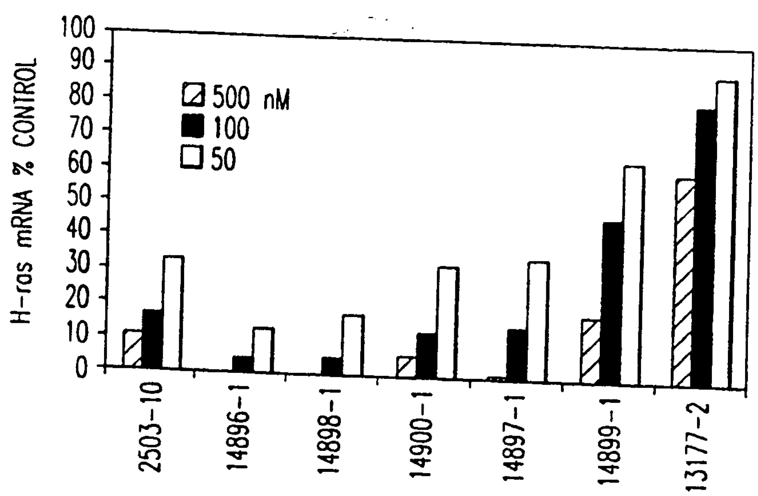


FIG. 23B

ISIS 2503	TCCGTCATCGCTCCTCAGGG	P=S
ISIS 13177	TTAGTAATAGCCCCACATGG	Scrambled control, P=S
ISIS 13920	TCCGTCATCGCTCCTCAGGG	MOE, P=S
ISIS 14896	TCCGTCATCGCTCCTCAGGG	MMI(1+1), P=S
ISIS 14898	TCCGTCATCGCTCCTCAGGG	MMI(2+2), P=S

P=S =phosphorothioate; MOE =methoxy ethyl;

FIG. 24A

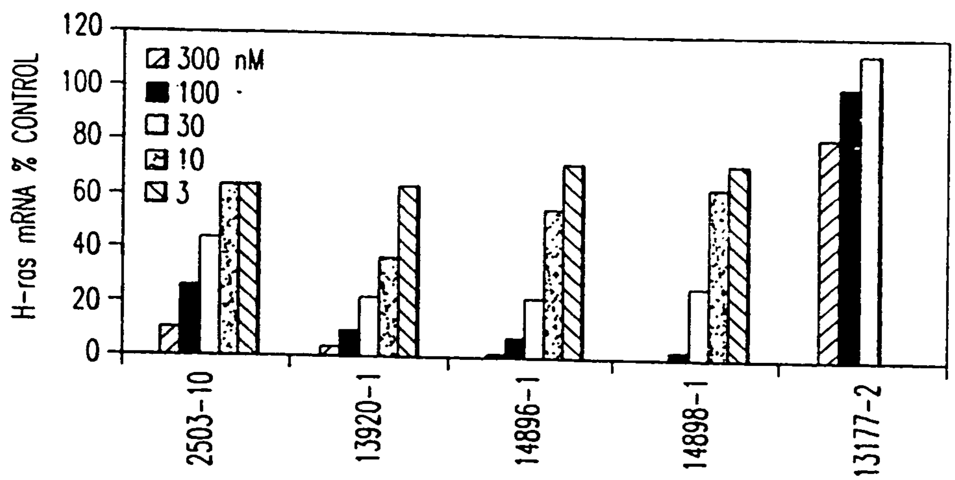


FIG. 24B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08800

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1; 536/27.21, 27.6, 27.8, 27.81, 28.4, 28.5, 28.53, 28.54, 24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	VASSEUR et al. Oligonucleosides: synthesis of a novel methylhydroxylamine linked nucleoside dimer and its incorporation into antisense sequences. J. American Chemical Society. 1992, Vol. 114, pages 4006-4007, see entire document.	1-4, 11, 12, 14, 15, 21-23, 28, 29 ---- 5-10, 13, 16-20, 24-27, 30-34
Y	US 5,623,070 A (COOK et al) 22 April 1997, see entire document.	1-34
Y	US 5,582,986 A (MONIA et al) 10 December 1996, see entire document.	1-34
Y	US 5,578,718 A (COOK et al) 26 November 1996, see entire document.	1-34

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 18 JUNE 1998	Date of mailing of the international search report 30 JUL 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dorthia Lawrence For JEFFREY FREDMAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08800

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SANGHVI et al. Concept, discovery and development of MMI linkage: story of a novel linkage for antisense constructs. Nucleosides & Nucleotides. 1997, Vol. 16, No. 7-9, pages 907-916, see entire document.	1-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08800

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/68; C12P 19/34; C07H 19/16, 19/167, 19/173, 19/067, 19/06, 19/09, 21/04; A61K 48/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 91.1; 536/27.21, 27.6, 27.8, 27.81, 28.4, 28.5, 28.53, 28.54, 24.5; 514/44

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAPLUS, CANCERLIT, BIOSIS, EMBASE, MEDLINE, DISSABS, LIFESCI, IFIPAT, NTIS
search terms: methylene, methylimino, chimeric, alternat?, DNA, RNA, nucleic, oligo, polynucleotide, oligonucleotide, PNA, peptide, protein

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